Volume 43 *May 1957* *Number 5*

Proceedings of the
National Academy of Sciences
of the United States of America

The Proceedings of the National Academy of Sciences

OFFICERS OF THE ACADEMY

DETLEV W. BRONK
President

GEORGE W. CORNER
Vice President

JOHN G. KIRKWOOD
Foreign Secretary

HUGH L. DRYDEN
Home Secretary

WILLIAM J. ROBBINS
Treasurer

EDITORIAL BOARD OF THE PROCEEDINGS

WENDELL M. STANLEY
Chairman

HUGH L. DRYDEN, *Home Secretary*
WILLIAM J. ROBBINS, *Treasurer*

JOHN G. KIRKWOOD, *Foreign Secretary*
EDWIN B. WILSON, *Managing Editor*

J. W. BEAMS	G. W. BEADLE	GREGORY BREIT
R. E. CLELAND	E. A. DOISY	L. C. DUNN
W. W. RUBEY	G. C. EVANS	W. F. LIBBY
HARLOW SHAPLEY	C. H. GRAHAM	J. VON NEUMANN
F. E. TERMAN	J. T. PATTERSON	PAUL WEISS

Subscription price is $12.50 for 1 year, $24.00 for 2 years, and $34.50 for 3 years; the price of a single issue is $1.50. Make all remittances payable to The University of Chicago Press in U.S. currency or its equivalent. Authorized agent for the British Commonwealth, except North America and Australasia: Cambridge University Press, Bentley House, 200 Euston Road, London, N. W. 1, England.

Editorial correspondence should be addressed to the PROCEEDINGS of the NATIONAL ACADEMY OF SCIENCES, Attention Miss Mary D. Alexander, University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois.

Business correspondence should be addressed to the Journals Division, University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois.

Subscribers are requested to notify The University of Chicago Press and their local postmaster immediately of change of address.

Microfilms of complete volumes of this journal are available to regular subscribers only and may be obtained at the end of the volume year from University Microfilms, 313 N. First Street, Ann Arbor, Michigan.

Entered as second-class matter June 21, 1920 at the Post-office at Easton, Pennsylvania, under the Act of August 24, 1912. Accepted for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917. Authorised June 18, 1920.

PRINTED IN THE U. S. A.

THE PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
is published for
THE NATIONAL ACADEMY OF SCIENCES
by
THE UNIVERSITY OF CHICAGO PRESS

Proceedings of the
NATIONAL ACADEMY OF SCIENCES

Volume 43 · Number 5 · May 15, 1957

*THE OXIDATION OF REDUCED TRIPHOSPHOPYRIDINE NUCLEOTIDE AS MEDIATED BY THE TRANSHYDROGENASE REACTION AND ITS INHIBITION BY THYROXINE**

By Eric G. Ball and Octavia Cooper

DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL, BOSTON, MASSACHUSETTS

Communicated March 25, 1957

Introduction.—The question of the interaction of the pyridine nucleotides in the manner described by the equation

$$\text{TPNH} + \text{DPN} \rightleftharpoons \text{DPNH} + \text{TPN}\dagger$$

was raised by one of us in 1941.[1] It was postulated then that a flavoprotein might serve to catalyze such a reaction. Some ten years later Colowick *et al.*[2] described an enzyme in *Pseudomonas fluorescens* catalyzing this reaction, and named it "transhydrogenase." The presence of transhydrogenase in animal tissues was shown a year later by Kaplan *et al.*[3] Whether or not the enzyme is a flavoprotein remains to be determined. We report data here to show that a purified particulate preparation from heart muscle which contains all the components of the cytochrome electron transmitter system also contains transhydrogenase. In the presence of this enzyme preparation, succinate and DPNH react rapidly with oxygen, but no reaction whatsoever occurs with TPNH, whether added as such or generated by the glucose-6-phosphate dehydrogenase system. Oxidation of TPNH does occur if a small amount of DPN is added. The inhibiting action of thyroxine on this oxidation of TPNH is also described, and the results are discussed in relation to the in vivo action of thyroxine.

Methods and Materials.—The procedure for making the beef heart muscle preparation which is employed has been described previously.[4] The pyridine nucleotides used were purchased either from Sigma Chemical Company or from Pabst Laboratories. Glucose-6-phosphate dehydrogenase, cytochrome c, glucose-6-phosphate, and L-thyroxine were Sigma preparations. Oxygen consumption was determined by the conventional Warburg manometric method, and a Beckman spectrophotometer, Model DU, equipped with a water-jacketed chamber was employed for spectrophotometric measurements. Other details of the individual experiments are given with the figures or in the text.

Results.—The ability of the heart muscle preparation to catalyze the oxidation of DPNH but not of TPNH is shown by the data presented in Figure 1. Here, as can be seen from the readings during the first 10-minute period, no oxidation either of TPNH or of DPNH occurs in the absence of the heart muscle preparation. On

tipping the enzyme into the flask containing DPNH, a rapid uptake of oxygen occurs. No oxygen uptake occurs, however, on tipping the enzyme into the vessel containing TPNH. At 40 minutes from the start of the experiment, 0.5 mg. of oxidized DPN was tipped into the flask containing TPNH. An immediate uptake of oxygen ensued. The control flask which showed no oxygen uptake contained 0.5 mg. of DPN and enzyme but no reduced pyridine nucleotides. The logical explanation of these results is the assumption that the heart muscle preparation con-

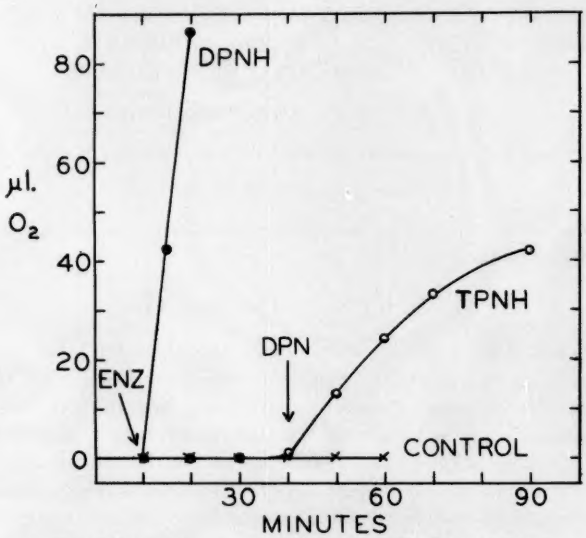


FIG. 1.—The oxidation of DPNH and TPNH. Each experimental Warburg vessel contained, at the start, 2.0 ml. of 0.1 *M* PO_4 buffer, pH 7.4; 0.1 ml. of cytochrome c, 6×10^{-4} *M*; 8 mg. (approximately 10 μmoles) of DPNH or TPNH; and H_2O. After 10 minutes 0.1 ml. of heart muscle preparation was tipped from one side arm. To the vessel containing TPNH, 0.5 mg. of DPN (in 0.1 ml. of H_2O) was tipped from a second side arm after 40 minutes. The final volume in each vessel was 3.0 ml. The control vessel contained no TPNH or DPNH, but all other components, including 0.5 mg. of DPN. Temperature, 37.2° C. The heart muscle preparation employed in this experiment had a dry weight of 4.2 mg./ml. Its nitrogen content was 12.9 per cent; phosphorus, 1.63 per cent. Under the same experimental conditions as used here, its activity per milligram of dry weight with succinate as substrate (final concentration 0.033 *M*) was 880 μl. O_2 per hour, and with paraphenylenediamine as substrate (final concentration 0.04 *M*), 1,100 μl. O_2 per hour.

tains transhydrogenase. It would also appear from the relatively slow rate of oxidation of TPNH as compared to that of DPNH that the rate-limiting step in the oxidation of TPNH is the transhydrogenase reaction. It should be noted that in other experiments of this type, where a different sample of TPNH was employed, a very slow uptake of O_2 in the absence of added DPN was encountered on addition of the enzyme. We attribute these results to the presence of small amounts of DPN in this particular TPNH sample. Since cytochrome c is present in excess in

these vessels, it would also appear from these results that this particular heart muscle preparation does not contain the specific TPNH–cytochrome c reductase described by Horecker[5] as occurring in pig liver.

In Figure 2 are shown the results of an experiment in which TPNH was generated by the glucose-6-phosphate dehydrogenase system and varying amounts of DPN were added. Again no oxygen uptake occurs in the presence of the heart muscle preparation with TPNH freshly generated by the glucose-6-phosphate dehydrogenase system until DPN is added. The rate of oxygen uptake then is a function of the amount of DPN present within the limits shown here.

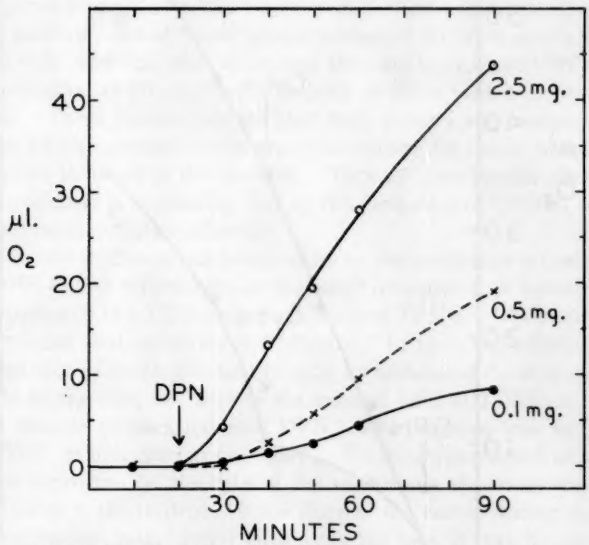


FIG. 2.—The effect of DPN concentration on the rate of oxidation of TPNH. Each Warburg vessel contained, in a final volume of 3.0 ml., glycylglycine buffer, 0.1 *M*, pH 7.4; glucose-6-phosphate dehydrogenase (Sigma, 690 K units/gm), 1.0 mg.; glucose-6-phosphate, 0.008 *M;* $MgCl_2$, 0.01 *M;* TPN, 2.7×10^{-4} *M* (0.66 mg.); heart muscle preparation, 0.1 ml.; and the indicated amounts of DPN, in 0.25 ml. of H_2O, tipped from the side arm after 20 minutes. Temperature, 37.2° C. A control vessel containing 0.5 mg. DPN and all other components except TPN showed no oxygen uptake.

If the oxidation of TPNH is proceeding in these experiments by means of the transhydrogenase reaction, then the flow of electrons through the heart muscle preparation to oxygen should be characteristic of that for the oxidation of DPNH by this preparation. Now it has been shown that the oxidation of DPNH by this same heart muscle preparation is dependent on the presence of cytochrome c.[6] The experiment portrayed in Figure 3 indicates that cytochrome c is also essential for the oxidation of TPNH. In order to demonstrate this dependence of the reaction on cytochrome c, it is necessary to deplete the heart muscle preparation of its intrinsic cytochrome c. This may be done by centrifuging the enzyme at 25,000 × *g* for 1 hour, homogenizing the enzyme pellet which forms with a buffered 0.5 per

cent deoxycholate solution, and recentrifuging at 25,000 × *g* for 1 hour.[6] The pellet so obtained is suspended in a volume of buffer equal to the volume of original enzyme preparation from which it was obtained. In Figure 3, curve *2* was obtained using such an enzyme preparation. As may be seen, the addition of cytochrome c stimulates the oxidation of TPNH by this enzyme preparation in the presence of DPN.

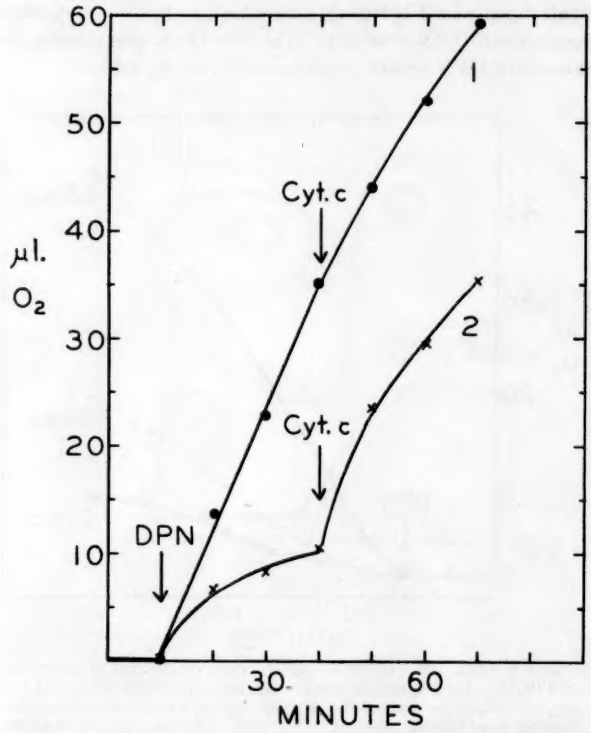


FIG. 3.—The effect of cytochrome c on TPNH oxidation. Each Warburg vessel contained, in a fluid volume of 3.0 ml., glycylglycine buffer, 0.15 *M*, pH 7.4; glucose-6-phosphate dehydrogenase, 1 mg.; glucose-6-phosphate, 0.008 *M*; $MgCl_2$, 0.01 *M*; TPN, 2.7×10^{-4} *M*; and 0.1 ml. of enzyme. The enzyme used for curve *1* was the original heart muscle preparation; for curve *2*, an equivalent amount of the heart muscle preparation which had been treated with 0.5 per cent Na deoxycholate (see text) was employed. DPN, 2.5 mg. in 0.1 ml. of H_2O, was tipped from one side arm after 10 minutes; cytochrome c to give a final concentration of 2×10^{-5} *M*, from a second side arm after 40 minutes. Temperature, 37.2° C.

As shown by curve *1* of Figure 3, the addition of cytochrome c to the vessel containing the untreated heart muscle preparation does not affect the rate of O_2 uptake. There is thus apparently sufficient intrinsic cytochrome c present in the original heart muscle preparation to prevent this component from being a rate-limiting factor in this reaction. As shown elsewhere,[6] the concentration of cytochrome c in the enzyme particles is of the order of 3×10^{-5} *M*. One treatment with

0.5 per cent deoxycholate solution serves to remove most, if not all, of this cytochrome c. Some of the succinate dehydrogenase and DPN–cytochrome c reductase, and over 50 per cent of the phospholipide, is also removed by this treatment.[6, 7] Therefore, the data presented in Figure 3, besides indicating the need for cytochrome c, also indicate that the deoxycholate treatment has not seriously depleted the transhydrogenase activity of the preparation. This enzyme thus appears to be fairly tightly bound to the particulate matter of this preparation, as are cytochromes c_1, b, a, and a_3.[6]

Though not shown in Figure 3, two other vessels were also run with exactly the same components present as in the vessel with the untreated enzyme preparation. In addition, however, one of these vessels contained KCN to give a final concentration of 10^{-3} *M*, and the other contained the naphthoquinone[8] SN 5949 to give a final concentration of 10 mg/liter. In both of these vessels no oxygen uptake was observed. These results indicate that both cytochrome oxidase and the unknown factor which operates in the electron transmitter chain somewhere below cytochrome c are involved in the reaction. Thus all these results strongly suggest that TPNH oxidation is proceeding first by the formation of DPNH, which is then oxidized by the main oxidative pathway.

In view of recent studies in our laboratories on the inhibitory action of thyroxine on certain DPN-linked dehydrogenase systems,[9] it appeared of interest to test the action of thyroxine on this DPN-linked oxidation of TPNH. The results presented in Figure 4 indicate that thyroxine is inhibitory. In these experiments the rate of oxidation was followed spectrophotometrically by measuring the decrease in density at 340 mμ due to the disappearance of the reduced form of TPNH in the presence of the heart muscle preparation and DPN. The reaction was started by the addition of DPN, as indicated in the figure. The inhibitory effect of varying concentrations of thyroxine on the rate of the reaction is shown by curves *2–4*, as compared to curve *1*, the control. If the slope of the curves during the initial 2–3 minutes of the reaction is employed to measure the rate, it may be calculated that in curve *4*, where the final concentration of thyroxine is 4.5×10^{-5} *M*, an 86 per cent inhibition has occurred. Correspondingly, the inhibitions in the case of curves *2* and *3* are 30 and 63 per cent, respectively, for final concentrations of thyroxine of 4.5×10^{-6} and 1.5×10^{-5} *M*. It should be emphasized that no proof is offered by this experiment that the site of action of thyroxine is upon the enzyme transhydrogenase. Indeed, we have obtained some evidence that thyroxine may inhibit to some extent the oxidation of DPNH by this heart muscle preparation. However, experiments to date which have been devised to elucidate more closely the site of action of thyroxine upon the oxidation of TPNH have not yet afforded an unequivocal answer.‡

Discussion.—The purified heart muscle preparation employed here is comprised of insoluble particles. Electron micrographs taken in collaboration with Dr. Russell Barrnett indicate that it is comprised largely of mitochondrial membranes. Earlier studies[4, 10–12] have shown that it is rich in phospholipide, contains flavin and cytochromes a, a_3, c, c_1, and b, and actively catalyzes the reaction of both succinate and DPNH with oxygen. Unpublished studies have shown that it is weakly active in catalyzing the oxidation of β-hydroxybutyrate if DPN is added. The results presented here indicate that the direct oxidation of TPNH by this integrated com-

plex of enzymes does not occur. Apparently, in order for electrons to pass from TPNH to oxygen over this network of electron carriers, TPNH must first hand them over to DPN by means of the enzyme transhydrogenase. The flow of electrons then apparently proceeds over the same members of the respiratory chain which are active in the oxidation of DPNH or succinate, since the reaction requires cytochrome c and is inhibited by cyanide and the naphthoquinone SN 5949.

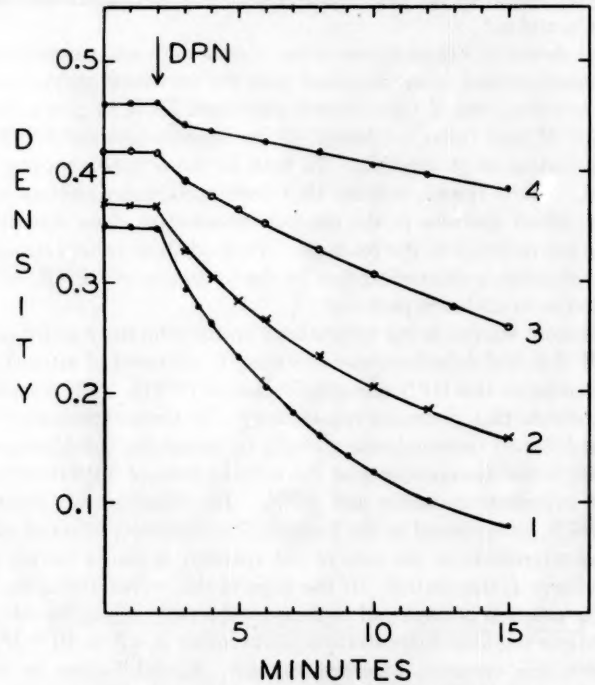


FIG. 4.—The inhibitory effect of thyroxine on TPNH oxidation. The rate of oxidation of TPNH was measured spectrophotometrically by measuring the decrease in absorption at wave length 340 mμ in a Beckman Model DU spectrophotometer at 36.5° ± 0.5° C., light path 1 cm. Each cuvette contained, in a total volume of 3.0 ml., glycylglycine buffer, 0.1 *M*, pH 7.4; 0.1 ml. of heart muscle preparation; 0.2 mg. of TPNH; 0.3 ml. of 10^{-3} *M* NaOH ± L-thyroxine; and 0.05 mg. of DPN, in 0.1 ml. of H_2O, added after 2 minutes to start the reaction. Curve *1:* control; curve *2:* thyroxine, $4.5 + 10^{-6}$ *M;* curve *3:* thyroxine, 1.5×10^{-5} *M;* curve *4:* thyroxine, 4.5×10^{-5} *M*, final concentration. Absorption due to the turbidity of the heart muscle preparation was balanced out by adding an equivalent amount of enzyme to the blank cuvette. The differences in initial absorption are due to thyroxine, which absorbs in this region.

An alternate route to the transhydrogenase pathway for the oxidation of TPNH in the animal body would appear to be over the specific TPNH–cytochrome c reductase.[5] Though this flavoprotein is described as being associated with particulate matter in the cell, it does not appear to be attached to the particles of the heart muscle preparation employed here. In mouse liver, Hogeboom and Schnei-

der[13] have shown that this enzyme is present in both the mitochondria and the microsomes. The distribution of transhydrogenase in the cell has not been established, to the best of our knowledge. The experiments of Kaplan *et al.*[14] indicate that it is present in the mitochondria, and the results presented here suggest that it is firmly attached to the mitochondrial membrane.

The observation made here that thyroxine inhibits the in vitro oxidation of TPNH via the transhydrogenase mechanism raises the question: Does thyroxine have a similar action in the animal body, and, if so, what might some of the consequences be? First, if the transhydrogenase pathway is impeded by thyroxine in vivo, it would seem likely that either oxidative processes requiring TPN would be retarded or there would have to be an increase in the amount of TPNH oxidized by the alternate pathway involving TPNH–cytochrome c reductase. Now Phillips and Langdon[15] have recently reported that the activity of TPNH–cytochrome c reductase in the liver of hyperthyroid rats is doubled. This finding, coupled with the earlier one of Drabkin[16] that the cytochrome c content of various tissues of the hyperthyroid rat is also increased, provides evidence to suggest that in the hyperthyroid state more TPNH is indeed oxidized by way of the TPNH–cytochrome c reductase pathway. Such findings suggest that inhibition of transhydrogenase by thyroxine may occur in vivo. It should be pointed out, however, that similar changes might be induced by an increase in metabolic processes proceeding over TPN-linked oxidations. For example, it has been shown by Glock and McLean[17] that there is a marked increase in the activity of both glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase in the liver of hyperthyroid rats. Both of these enzymes are TPN-specific, and thus increases in their activities might well trigger corresponding changes in the activities of TPN–cytochrome c reductase and cytochrome c. Another consequence of an inhibition in vivo by thyroxine of TPNH oxidation by the transhydrogenase pathway is suggested by the results of Kaplan *et al.*[14] These workers have reported that in isolated liver mitochondria the oxidation of TPNH by pathways other than that involving transhydrogenase yields little high-energy phosphate. Thus inhibition of transhydrogenase activity by thyroxine could be expected to result in a lower yield of high-energy phosphate for each oxygen molecule consumed. Under such circumstances the animal's total oxygen consumption would rise above the normal. Experiments are under way which, it is hoped, will cast some light on the possible relationship of these various aspects to the inhibitory action of thyroxine on transhydrogenase.

Summary.—A purified preparation from heart muscle which contains all the components of the cytochrome electron transmitter system is shown to contain transhydrogenase. In the presence of this enzyme preparation succinate and DPNH react rapidly with oxygen, but no reaction whatsoever occurs with TPNH, whether it is added as such or is generated by the glucose-6-phosphate dehydrogenase system. Oxidation of TPNH does occur if a small amount of DPN is added. This DPN-mediated oxidation of TPNH requires cytochrome c and is inhibited by cyanide and the naphthoquinone SN 5949. The pathway of electron flow is thus characteristic of that for the oxidation of DPNH or succinate by the same enzyme preparation. L-Thyroxine is shown to inhibit the oxidation of TPNH by this pathway, and some of the possible implications of this finding for the mode of action of thyroxine in vivo are discussed.

* This work was supported in part by funds received from the Eugene Higgins Trust through Harvard University and from the Life Insurance Medical Research Fund.

† The following abbreviations are used: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide respectively; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide respectively.

‡ Recent experiments have clearly indicated that thyroxine does inhibit the transhydrogenase reaction. It has also been possible to show that the naphthoquinone SN5949 does not exert its inhibitory effect by a direct action upon the transhydrogenase enzyme.

[1] E. G. Ball, in *A Symposium on Respiratory Enzymes* (Madison: University of Wisconsin Press, 1942), p. 38.

[2] S. P. Colowick, N. O. Kaplan, E. F. Neufeld, and M. M. Ciotti, *J. Biol. Chem.*, **195**, 95, 1952.

[3] N. O. Kaplan, S. P. Colowick, and E. F. Neufeld, *J. Biol. Chem.*, **205**, 1, 1953.

[4] E. G. Ball and O. Cooper, *J. Biol. Chem.*, **180**, 113, 1949.

[5] B. F. Horecker, *J. Biol. Chem.*, **183**, 593, 1950.

[6] E. G. Ball and O. Cooper, *J. Biol. Chem.* (in press).

[7] E. G. Ball, in *Henry Ford Hospital Symposium: Enzymes, Units of Biological Structure and Function* (New York: Academic Press, Inc., 1956), p. 433.

[8] E. G. Ball, C. B. Anfinsen, and O. Cooper, *J. Biol. Chem.*, **168**, 257, 1947.

[9] E. C. Wolff and E. G. Ball, *J. Biol. Chem.*, **224**, 1083, 1957.

[10] E. G. Ball and O. Cooper, *J. Biol. Chem.*, **198**, 629, 1952.

[11] E. G. Ball, C. F. Strittmatter, and O. Cooper, *J. Biol. Chem.*, **193**, 635, 1951.

[12] S. W. Edwards and E. G. Ball, *J. Biol. Chem.*, **209**, 619, 1954.

[13] G. H. Hogeboom and W. C. Schneider, *J. Biol. Chem.*, **186**, 417, 1950.

[14] N. O. Kaplan, M. N. Swartz, M. E. Frech, and M. M. Ciotti, these PROCEEDINGS, **42**, 481, 1956.

[15] A. H. Phillips and R. G. Langdon, *Biochim. et biophys. acta*, **19**, 380, 1956.

[16] D. L. Drabkin, *J. Biol. Chem.*, **182**, 335, 1950.

[17] G. E. Glock and P. McLean, *Biochem. J.*, **61**, 390, 1955.

---

*INTERRELATIONSHIP BETWEEN VITAMIN E AND LIPIDE COFACTOR IN THE CYTOCHROME C REDUCTASE SYSTEM**

BY KENNETH O. DONALDSON† AND ALVIN NASON

MCCOLLUM-PRATT INSTITUTE, THE JOHNS HOPKINS UNIVERSITY, BALTIMORE, MARYLAND

Communicated by B. H. Willier, March 13, 1957

Although the importance of lipides in the oxidation of reduced diphosphopyridine nucleotide (DPNH) and succinate via the cytochrome system has been well established,[1–8] their mode of action has remained somewhat obscure. It has already been demonstrated that added vitamin E (the tocopherols) increases the activity of the diphosphopyridine nucleotide (DNP)- and succinate-cytochrome c reductases from rat skeletal muscle and bovine heart muscle; and that the almost complete loss of enzymatic activity resulting from iso-octane extraction can be completely restored by the addition of tocopherol or, even more effectively, by the lipide residue obtained from vacuum distillation of the iso-octane after extraction. It has also been shown that the added tocopherol acts in a catalytic fashion in the sequence of electron transport just before the cytochrome c position and that there is a competitive inhibition of antimycin A with tocopherol.[6–8]

The present paper reports new experiments demonstrating that the crude lipide residue obtained by iso-octane extraction of the cytochrome c reductases from rat

skeletal muscle and bovine heart muscle can be shown to contain vitamin E, most of it as a presumed tocopheryl quinone, in amounts too small, however, to account for enzyme reactivation. The active compound of the crude lipide residue, designated as the "lipide cofactor," has been purified and tentatively identified as a mixed triglyceride. Further evidence is presented which suggests that added lipide cofactor may be acting indirectly by releasing endogenous vitamin E of the preparation to the "active sites" of the enzyme.

Methods and Materials.—The particulate cytochrome c reductases from rat skeletal muscle and bovine heart muscle were prepared and assayed as already described.[7] Tocopherol,[9] other lipides, and the lipide cofactor were used in the enzymatic reaction mixtures as the usual bovine serum albumin suspensions.[8] Iso-octane extraction of the enzymes was performed as previously indicated.[8] Tocopherol was determined colorimetrically by a modification of the iron chloride–bipyridyl method of Emmerie and Engel[10] and confirmed to be α-tocopherol by the paper-chromatographic procedure of Green *et al.*[11] The conversion of tocopheryl quinone to tocopherol by means of an HCl–ascorbic acid reduction procedure has recently been described by Harrison *et al.*[12] This procedure was used in the present work to convert a naturally occurring substance, presumably tocopheryl quinone, to α-tocopherol in the nonsaponifiable fraction of crude lipide obtained from the above enzyme preparations.

Results and Discussion.—Tocopherol is now found in the nonsaponifiable fraction of the concentrated crude residue obtained by iso-octane extraction of large quantities of rat muscle enzyme (Table 1). Most of it is present presumably as the

TABLE 1

TOCOPHEROL CONTENT OF PARTICULATE ENZYME PREPARATIONS FROM RAT SKELETAL MUSCLE AND BOVINE HEART MUSCLE

(μM Tocopherol/Gm Protein)

	Iso-octane Extracted		Remaining*		
Preparation	Before Reduction	After HCl–Ascorbic Acid Reduction	Before Reduction	After HCl–Ascorbic Acid Reduction	Total†
Rat skeletal muscle enzyme:					
1	0.18	0.42	0.82	4.08	4.5
2	0.08	0.16	0.75	4.94	5.1
3	0.18	0.90	1.55	4.30	5.2
Bovine heart muscle enzyme:					
1	1.33	1.60	4.44	8.40	10.0
2	1.88	1.65	5.68	9.65	11.3
3	1.55	1.57	3.70	8.43	11.0

* The tocopherol remaining in the enzyme preparations after iso-octane extraction was determined in the non-saponifiable fraction of the total lipide (see Nason and Lehman, *J. Biol. Chem.*, **222**, 511, 1956) by the procedures already indicated under "Methods and Materials."

† Calculated by summation of the values for "Iso-octane Extracted" and "Remaining" tocopherol after HCl–ascorbic acid reduction.

tocopheryl quinone which can be reduced to tocopherol by the HCl–ascorbic acid procedure, as already demonstrated by Bouman and Slater[13] for a Keilin-Hartree heart muscle preparation. Calculations of the data of Table 1 show that only 3–17 per cent of the total tocopherol of the rat skeletal muscle enzyme is removed by iso-octane extraction. This occurs with a concomitant loss of approximately 90 per cent of the cytochrome c reductase activity. As much as 85 per cent of the total tocopherol in the enzyme appears to be present in the presumed tocopheryl quinone

form. Iso-octane extraction removes only 10–20 per cent of the tocopherol form. The ratio of tocopherol to presumed quinone present in the corresponding bovine heart muscle enzyme is larger when compared to that of the rat skeletal muscle system. In most preparations virtually all the vitamin E removed from the bovine heart muscle enzyme by iso-octane extraction is in the tocopherol form and represents only 15 per cent of the total vitamin E in the enzyme preparation. Occasionally, however, a bovine heart muscle preparation was obtained which was similar to that of rat muscle with respect to the ratio of tocopherol to the presumed quinone. Of the vitamin E remaining (85 per cent) in the iso-octane-extracted bovine heart muscle enzyme, about half is present as tocopherol and the other half as the presumed tocopheryl quinone. The bovine heart muscle system contains twice as much vitamin E as the rat skeletal muscle enzyme.

Previous inability to demonstrate the presence of tocopherol in the crude lipide residue obtained by iso-octane extraction of the rat skeletal muscle enzyme can be attributed to a combination of circumstances: (1) most of the vitamin E is present in the presumed tocopheryl quinone form and could be detected only by converting it to tocopherol through the recently described HCl–ascorbic acid reduction method,[12] and (2) relatively small quantities of enzyme (15–50 ml.) were formerly used for iso-octane extraction. However, by using large quantities of rat muscle enzyme (100–500 ml.), it is now possible to measure tocopherol in the lipide residue obtained by iso-octane extraction. Thus in a typical experiment the quantity of crude lipide residue obtained by iso-octane extraction of particulate rat skeletal muscle enzyme which restored 50 per cent of the activity of the extracted cytochrome c reductase contained only 0.03 μg. of tocopherol even after HCl–ascorbic acid reduction. It has already been established[8] that 45–50 μg. of *d*-α-tocopherol are necessary for 50 per cent reactivation under the same conditions. Therefore, enzymatic reactivation cannot be ascribed to the tocopherol content of the residue. Instead, reactivation is due to the presence of a lipide cofactor in the residue.

The lipide cofactor of the crude residue obtained by iso-octane extraction and responsible for restoration of cytochrome c reductase activity has been purified 200-fold from bovine heart muscle homogenate. The purified lipide is about 20 times as active as tocopherol, mole for mole, and does not show the presence of tocopherol even after HCl–ascorbic acid treatment. Alkaline hydrolysis completely destroys the activity of the lipide cofactor, whereas it leaves that of *d*-α-tocopherol unchanged. By means of infrared analysis, characterization of the chemical derivatives, and chromatographic and countercurrent distribution techniques the lipide cofactor has been tentatively identified as a mixed triglyceride with stearate, palmitate, and oleate components. Of the more than one hundred different substances tested for restoration of activity,[8] including steroids, mono-, di-, and triglycerides and other esters, phospholipides, fat-soluble vitamins, and antioxidants, approximately ten of these showed significant restoration of activity. These included milk, butter, oleomargarine, synthetic corn oil, triolein, ethyl stearate, *n*-butyl stearate, and certain synthetic glycerides.

Two facts are of interest: (1) iso-octane extraction removes only about 10 per cent of the total tocopherol of the particulate cytochrome c reductase system, with a concomitant loss of 90 per cent of enzymatic activity; and (2) structurally dissimilar molecules such as the tocopherols and certain triglycerides and long-chain

fatty acid esters are effective in restoring activity of the iso-octane-extracted enzyme. Apparently the small quantity of tocopherol in the crude lipide residue cannot account for enzymatic restoration. However, it is possible that before iso-octane extraction it was originally effective at the "active enzyme sites." The possibility also exists that addition of the lipide cofactor or of certain other effective esters replenishes the "active centers" of the extracted enzyme by releasing some of the remaining tocopherol, perhaps from a "bound" form. This would be tantamount to adding tocopherol to the system. The activity of these effective esters could thus be attributed directly to tocopherol. A partial test of this hypothesis would be an examination of the effects of various lipides in potentiating the removal of tocopherol from the enzyme by iso-octane extraction. Table 2 shows

TABLE 2*

EFFECT OF VARIOUS LIPIDES ON THE SUBSEQUENT RELEASE OF TOCOPHEROL BY ISO-OCTANE EXTRACTION OF BOVINE HEART ENZYME

SUBSTANCES	EFFECT IN RESTORATION OF ISO-OCTANE-EXTRACTED ENZYME	μM OF TOCOPHEROL EXTRACTED— Before Reduction	After HCl–Ascorbic Acid Reduction
EtOH-albumin (control)	Inactive	0.07	0.33
Glycerol dipalmitate	Inactive	0.09	0.36
Tristearin	Inactive	0.09	0.35
Purified lipide cofactor (from bovine heart muscle)	Active	0.16	0.86
Oleomargarine	Active	0.27	1.09
n-Butylstearate	Active	0.14	0.65

* 1.5 gm. of each substance were homogenized with 20 ml. of ethanol–bovine serum albumin in a Ten Broeck homogenizer and added to 100 ml. each of particulate bovine heart enzyme in two separate portions. After each addition, the enzyme was extracted twice with an equal volume of cold iso-octane in the usual manner (see Nason and Lehman, *J. Biol. Chem.*, **222**, 511, 1956). The combined iso-octane extracts were evaporated *in vacuo*, the lipide residue taken up in absolute ethanol, and tocopherol determined in the nonsaponifiable faction as indicated.

that the addition of the lipide cofactor or of other indicated active substances to the bovine heart muscle enzyme results in a two- to threefold release of tocopherol and presumed tocopheryl quinone, as compared with the control, upon subsequent extraction with iso-octane. Inactive compounds such as glycerol-dipalmitate and tristearin are ineffective in potentiating the removal of tocopherol by subsequent iso-octane extraction. Glycerol monostearate (not shown), which is inactive, was also ineffective in this respect. It is also worthy of note that treatment of the enzyme with lipide cofactor followed by iso-octane extraction yields a lower cytochrome c reductase activity as compared with the control. The addition of lipide cofactor and α-tocopherol, separately or in various combinations, in most cases did not restore enzymatic activity. Morrison *et al.*,[14] using iso-octane-extracted succinate cytochrome c reductase from pig heart have recently confirmed the reactivating effect of *d*-α-tocopherol and the lipide residue derived from iso-octane extraction. Reactivation was also effected by a hemin-lipide complex isolated from heart muscle. It is not unlikely that the hemin-lipide might be acting like the other active compounds in potentiating the mobility of endogenous tocopherol as suggested above.

The present experiments offer further support for the function of vitamin E as a cofactor in the cytochrome c reductase system.[6, 8] Moreover, they provide a reasonable explanation for the fact that such structurally unlike molecules as the tocopherols and certain triglycerides and long-chain fatty acid esters can restore enzyme activity. The new findings that some vitamin E is removed by iso-octane

extraction, while most of it still remains in the enzyme, and that only those lipides which are effective in reactivation of cytochrome c reductase also potentiate the mobility or extractability of endogenous tocopherol point to a primary role for the vitamin. The additional observation that a gradual removal of tocopherol from the enzyme system results in a gradual loss of activity lends further strength to this suggestion. It is also of interest that tocopherol has been reported to be the only fat-soluble vitamin present in a Keilin-Hartree heart muscle preparation.[13] Nevertheless, the possibility of a more direct role of the lipide cofactor has not been excluded.

Thus far, it has not been possible to demonstrate that vitamin E functions directly as an electron carrier in the cytochrome c reductase system, although it has already been shown to function in a catalytic manner.[8] However, the possibility has not been eliminated that undetectable quantities of vitamin E bound to the "active enzyme sites" undergo oxidation-reduction changes. Chemical studies demonstrating reversible oxidation reactions of the vitamin tend to favor an electron-transport role in the enzyme system. Alternately, the possibility still exists that vitamin E may be functioning indirectly as a binding or cementing substance for other components of the cytochrome c reductase system. Work is now in progress in this laboratory to elucidate further the mechanism of action of vitamin E.

Summary.—By using large quantities of particulate cytochrome c reductase prepared from rat skeletal muscle and bovine heart muscle, it has been possible to show that the crude lipide residue obtained by iso-octane-extraction of the enzyme contains vitamin E, most of it as a presumed tocopheryl quinone. Iso-octane removes only 10–20 per cent of the total vitamin E, with a concomitant loss of 90 per cent of the cytochrome c reductase activity. The amount of vitamin E extracted, however, cannot account for enzyme reactivation by added crude lipide residue. Instead, restoration is attributed to a lipide cofactor tentatively identified as a mixed triglyceride with stearate palmitate and oleate components. It is possible, however, that the small quantity of vitamin E removed by iso-octane extraction may originally have been effective at the "active enzyme sites." The fact that the lipide cofactor and other active lipides potentiate the extractability of endogenous vitamin E is interpreted to suggest that they are acting by releasing the vitamin to the "active sites" of the enzyme. Whether the vitamin is functioning directly in electron transfer or indirectly as a binding substance remains to be established.

* Contribution No. 178 of the McCollum-Pratt Institute. This investigation was supported in part by a research grant from the Muscular Dystrophy Associations of America, Inc.

† Postdoctoral fellow of the McCollum-Pratt Institute.

[1] A. P. Nygaard and J. B. Sumner, *J. Biol. Chem.*, **200**, 723, 1953.

[2] S. W. Edwards and E. G. Ball, *J. Biol. Chem.*, **209**, 619, 1954.

[3] C. Martius, *Rapp. 3ème Congr. intern. biochim. Bruxelles Conf. Rapp.*, p. 1, 1955.

[4] E. Stotz, M. Morrison, and G. Marinetti, in *Henry Ford Hospital Symposium: Enzymes Units of Biological Structure and Function* (New York: Academic Press, Inc., 1956), p. 401.

[5] D. E. Green, in *Henry Ford Hospital Symposium: Enzymes, Units of Biological Structure and Function* (New York: Academic Press, Inc., 1956), p. 465.

[6] A. Nason and I. R. Lehman, *Science*, **122**, 19, 1955.

[7] I. R. Lehman and A. Nason, *J. Biol. Chem.*, **222**, 497, 1956.

[8] A. Nason and I. R. Lehman, *J. Biol. Chem.*, **222**, 511, 1956.

[9] *d*-α-tocopherol was generously supplied by the Distillation Products Division of the Eastman Kodak Company.

[10] A. Emmerie and C. Engel, *Rec. trav. chim.*, **57**, 1351, 1938.

[11] J. Green, S. Marcinkiewics, and P. R. Watt, *J. Sci. Food Agr.*, **6**, 274, 1955.

[12] W. H. Harrison, J. E. Gander, E. R. Blakley, and P. D. Boyer, *Biochim. et biophys. acta*, **21**, 150, 1956.

[13] J. Bouman and E. C. Slater, *Nature*, **177**, 1181, 1956.

[14] M. Morrison, R. Crawford, and E. Stotz, *Biochim. et biophys. acta*, **22**, 579, 1956.

---

*MEIOTROPHIC MUTANTS OF PASTEURELLA PESTIS AND THEIR USE IN THE ELUCIDATION OF NUTRITIONAL REQUIREMENTS**

BY ELLIS ENGLESBERG AND LAURA INGRAHAM

BIOLOGICAL LABORATORY, LONG ISLAND BIOLOGICAL ASSOCIATION, COLD SPRING HARBOR, NEW YORK

Communicated by M. Demerec, March 8, 1957

During the course of characterizing the natural breaks in the biosynthesis of amino acids by *Pasteurella pestis* and of isolating mutants requiring fewer growth factors than the prototroph (meiotrophic mutants[1]), it became apparent that this procedure of meiotrophic mutant isolation and characterization was of general value in the elucidation of the amino acid requirements of other fastidious heterotrophic[2] micro-organisms.

Pasteurella pestis, strain A1122B1, avirulent, and various meiotrophic mutants were employed in this study. Stock cultures were maintained on casein hydrolyzate mineral glucose agar. The complete synthetic medium employed had the following composition: glucose, 0.2 per cent; $MgSO_4(7H_2O)$, 0.05 per cent; $CaCl_2$, 10^{-3} per cent; $FeCl_3(6H_2O)$, 2.5×10^{-4} per cent; Na_2SO_3, 0.1 per cent; Na_2HPO_4-KH_2PO_4 (pH 7), $M/20$; agar (Bacto), 1.5 per cent; and the following amino acids in amounts to yield a 0.004 per cent solution: *l*-cystine (C), *dl*-methionine (M), *dl*-valine (V), *dl*-isoleucine (I), and *dl*-phenylalanine (P). The *l* forms of the amino acids can be substituted for the *dl*. The medium was prepared by autoclaving together agar, Na_2SO_3, and distilled water, and the other ingredients were added subsequent to autoclaving as sterile concentrated solutions. In some early experiments a smaller concentration of sulfite (0.05 per cent) was employed, and in some cases thiosulfate was used in place of cystine. Although 0.05 per cent sulfite was subsequently found to be inadequate to fully neutralize the inhibitory effect of agar on *P. pestis*,[3] it gave a medium highly sensitive to the detection of isoleucine and valine inhibitions. Agar autoclaved at acid pH was employed initially as another means of preventing the inhibitory effect of the agar on the growth of *P. pestis*.

Mutation rates to meiotrophy were determined by a modification of the Luria and Delbrück[4] procedure and were calculated by the median method of Lea and Coulson.[5]

Pasteurella pestis A1122B1 has been previously shown to require cysteine, phenylalanine, methionine, valine, and isoleucine for growth at 30° C. in a mineral glu-

cose medium.[6] These requirements appear to be those of the wild type.[7, 8] An examination of the cysteine, methionine requirement has demonstrated just two breaks in the biosynthesis of these amino acids: one in the conversion of sulfate to sulfite and the other in the conversion of cysteine to cystathionine. A methionine-independent mutant, *me*$^+$ (previously called *M*1 or *M*$^+$) was readily isolated from the prototroph on a thiosulfate-, valine-, isoleucine-, and phenylalanine-supplemented agar medium. Because of the absence of methionine, little if any growth of the prototroph occurs on this medium, and selection takes place for the methionine-independent mutants. (Since sulfite, thiosulfate, cysteine, and cystine are interchangeable, *me*$^+$ can be isolated with any of these compounds as the sole sulfur sources.)

With the isolation of *me*$^+$ the possibility of isolating mutants independent of the other amino acids was explored. Since valine and isoleucine are sometimes both required as a result of the loss of a common enzyme in their biosynthesis,[9] an attempt was made to select for a valine, isoleucine meiotroph in one step by streaking the prototroph onto a CPM medium. The prototroph failed to grow on this medium, and mutant colonies appeared after several days' incubation. The mutants were isolated and found to be extremely stable. Thus the "requirement" for valine and isoleucine was lost in one step. These results appeared to indicate that it would be possible to develop a mutant strain of *P. pestis* which would grow on CP alone, by a two-step mutation and selection procedure. When the valine, isoleucine meiotroph was streaked heavily onto CP agar, it failed to grow, and secondary mutant colonies were isolated which were methionine-, valine-, and isoleucine-independent, apparently confirming this supposition. However, an entirely different picture developed when the mutants were selected in the reverse manner. When the *me*$^+$ meiotroph isolated on CPVI or thiosulfate PVI was streaked onto CP, growth of the entire population occurred. Subsequently, methionine, valine, isoleucine meiotrophs were isolated directly from the prototroph on CP, and were similar, if not identical, to those mutants isolated on CPVI. That this was a single mutational event was demonstrated by the finding that the viable counts on CP and CPVI agar plates of portions of the secondary colonies isolated directly from the prototroph on CP and CPVI agar were the same and were equal to that on a CPMVI medium. Thus the valine, isoleucine, methionine "requirement" appeared to be lost simultaneously. It seemed, therefore, that in the selection of valine, isoleucine, methionine meiotrophs, if selection first occurred for a mutant able to grow in a medium lacking valine and isoleucine (CPM), a further mutational event was required for growth on CP, whereas if selection for such a mutant was made directly onto CP or CPVI agar, only one mutational event was required.

An explanation of these apparently contradictory findings was arrived at by the observation that *me*$^+$ (which grew on CP) are completely inhibited when streaked onto CPM and give rise to secondary colonies which are able to grow on this medium. Thus mutation on CPM plates is to methionine resistance (*me-r*), and the valine, isoleucine "requirement" of the prototroph is actually the result of the inhibitory effect of methionine. Thus the addition of valine and isoleucine to CPM "neutralizes" the methionine inhibition, allowing for the growth of the prototroph.

The process of selection of the double mutants *me*$^+$ *me-r* and the respective muta-

tion rates are outlined in Figure 1. The characteristic responses of these mutants as compared to the prototroph are shown in Table 1. Isoleucine and valine are

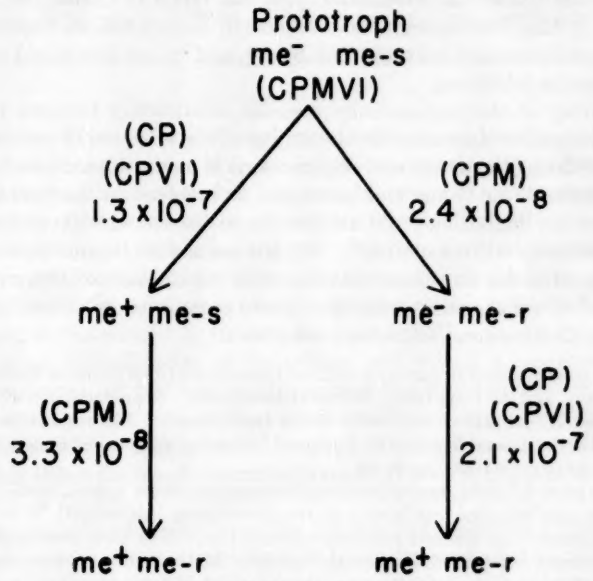


FIG. 1.—Mutation to methionine independence (*me*$^+$) and to methionine resistance (*me-r*). Letters in parentheses indicate the amino acid composition of the medium employed in selecting the mutants. The mutation rates given are based upon an average of two determinations.

TABLE 1*

GROWTH CHARACTERISTICS OF *Pasteurella pestis* A1122B1 PROTOTROPH AND VARIOUS MEIOTROPHIC MUTANTS

	AMINO ACID COMPOSITION OF MEDIA															
	CPMVI		CP		CPI		CPV		CPVI		CPM		CPMI		CPMV	
STRAINS	48†	96†	48	96	48	96	48	96	48	96	48	96	48	96	48	96
Prototroph	2	3	–	–	–	–	–	–	–	–	–	–	–	–	1	1
me$^+$	2	3	4	5	2	5	3	5	4	5	–	–	–	–	–	–
me-r	3	5	–	–	–	–	–	–	–	–	4	5	2	5	3	5
me$^+$ *me-r*	3	5	3	5	2	5	2	5	4	5	4	5	2	5	3	5

* Mineral glucose agar plates (agar autoclaved under acid conditions to prevent agar inhibition) were supplemented with the amino acids indicated and streaked with a light washed suspension of cells previously grown for 24 hours on a casein hydrolyzate mineral glucose slant. Plates were incubated at 30° C. and observed daily. – = no growth; 1, 2, 3, 4, and 5 represent degrees of growth, varying from 1 = just detectable growth to 5 = excellent growth. C = *l*-cystine, P = *dl*-phenylalanine, M = *dl*-methionine, V = *dl*-valine, I = *dl*-isoleucine. The two double mutants *me*$^+$ *me-r* isolated in reverse manner gave identical results.

† Time in hours.

inhibitory, as indicated by the comparison of growth in CP, CPI, and CPV media, and both these compounds when present together apparently "neutralize" the individual inhibitory effects. The inhibition by valine, and to some extent by isoleucine, is extremely variable from one experiment to another. In some cases

no inhibition by valine was noted. Analysis of several experiments indicates that the valine inhibition may be potentiated by slightly inhibitory agar. Under conditions in which valine was noninhibitory, it still failed to release the methionine inhibition. It therefore appears that isoleucine is required to "neutralize" the methionine inhibition and is in itself inhibitory, and valine is required to "neutralize" the isoleucine inhibition.

The discovery of the unexpectedly complex relationship between methionine, valine, and isoleucine demonstrates the utility of the selection of meiotrophic mutants for elucidating the amino acid requirements of heterotrophic micro-organisms. The "requirement" for valine and isoleucine, as involved in the "neutralization" of the methionine inhibition, could not have been discovered without first isolating a methionine-independent meiotroph. Thus it seems that this method of approach would be essential for any clear, unambiguous explanation of the growth-factor requirements of other, more fastidious micro-organisms, such as *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Clostridium*, and others.

* This work was supported in part by a contract between the Office of Naval Research, Department of the Navy, and the Long Island Biological Association. Reproduction in whole or in part is permitted for any purpose of the United States Government. Brief summaries of this work have appeared in the *Annual Report of the Biological Laboratory, Long Island Biological Association* (*1954–55*), pp. 23–27; (*1955–56*), pp. 26–28.

[1] *Pasteurella pestis* A1122B1, having prototrophic requirements for cystein, methionine, phenylalanine, valine, and isoleucine, can mutate in two directions in this respect: it can give rise to auxotrophic mutants (i.e., mutants requiring additional growth factors) or to meiotrophic mutants (i.e., mutants requiring fewer growth factors). The adjective "meiotrophic" and the noun "meiotroph" have been adopted to avoid the use of such ill-defined terms as "gain" mutation, "reverse" mutation, or "back" mutation, and since operationally it is not known whether mutation of naturally deficient strains leading to an ability to grow without a particular growth factor is the result of a gain of a new gene, a mutation to an allele previously active, a suppressor mutation, or a mutation away from some inhibition. See E. Englesberg and B. D. Davis, *Microbial Genetics Bull.*, **7**, 36, 1953; E. Englesberg, *J. Bacteriol.*, **68**, 57–60, 1954.

[2] The term "heterotrophic" is used here according to the definition in the Appendix to *Cold Spring Harbor Symposia Quant. Biol.*, **11**, 302–303, 1946.

[3] E. Englesberg, *J. Bacteriol.*, **73**, 1957.

[4] S. E. Luria and M. Delbrück, *Genetics*, **28**, 491–511, 1943.

[5] D. E. Lea and C. A. Coulson, *J. Genet.*, **49**, 264–285, 1949.

[6] E. Englesberg, *J. Bacteriol.*, **63**, 675–680, 1952.

[7] M. Rockenmacher, thesis, University of California, Berkeley, 1950.

[8] G. M. Hills and E. D. Spurr, *J. Gen. Microbiol.*, **6**, 64–73, 1952.

[9] D. Rudman and A. Meister, *J. Biol. Chem.*, **200**, 591–604, 1953.

*COMPETITIVE BINDING OF ATP AND ACRIDINE ORANGE BY MUSCLE**

By George Karreman, Helmut Mueller, and Albert Szent-Györgyi

INSTITUTE FOR MUSCLE RESEARCH, MARINE BIOLOGICAL LABORATORY, WOODS HOLE, MASSACHUSETTS

Communicated March 11, 1957

Introduction.—The lifetime of electronic singlet excitation in molecules is too short to allow the utilization of these excitational energies in biological systems.[1] Triplet excitations last longer, but the probabilities of their occurrence are small. Circumstances which increase the probability of these transitions may thus have biological importance. It has been shown[1] that dyes and certain other substances which are excited to singlets by ultraviolet light, in their dilute aqueous solution, go into triplets if the solution is frozen. This transition may be due to changes taking place in the solvent or in the solute, or in both. It can be assumed that freezing causes crystallization of the solvent and with it a consequent increased local concentration of the solute. Increasing the concentration of certain dyestuffs in aqueous solution may lead to reversible molecular aggregations in the form of dimers and polymers, which, on excitation, may tend to go into the triplet state. That this is the case for acridine orange has been demonstrated in particular by the work of Zanker.[2]

Acridine orange is especially suited for the study of these relations, since it shows a green fluorescence under ultraviolet light in singlet, and a red phosphorescence in triplet, excitation. Accordingly, a dilute aqueous solution (10^{-3}–10^{-4} *M*) shows a vivid green color, which turns gradually into red as the concentration of the dye is increased and polymers are formed.

The nature of the bond between two molecules of the acridine orange dimer can be decided by the behavior in organic solvents like alcohol or acetone, which will dissociate complexes held together by dispersion forces,[3] while they leave electropolar bonds unaffected. Accordingly, addition of alcohol or acetone to a concentrated aqueous solution of acridine orange causes a change in color from red to green, as observed under the ultraviolet lamp, dispersion forces being responsible for the bond holding the dimers together.

As is being shown in this laboratory, ATP forms a red phosphorescent complex with acridine orange, as does adenine, indicating that it is the purine part of the molecule which dimerizes with the dye. Thus the dye can also form complexes with molecules of a different substance. Dimers formed by two equal molecules are, in this laboratory, called "homodimers," while dimers formed by two different molecules are called "heterodimers."

If the psoas muscle of the rabbit, extracted with glycerol,[4] is washed out with water, then suspended in a 10^{-3} *M* acridine orange solution, and placed, after a while, on filter paper, it is found to show an intense red color if illuminated with a high-pressure mercury lamp, the visible light of which is eliminated by an appropriate filter. The dye seeping out into the underlying filter paper is green. This indicates that the dye has been bound by a muscle constituent in the form of a complex, a heterodimer, which goes into the triplet state on excitation. A drop of alcohol placed on the muscle fiber changes the red color into green, showing that dispersion forces underlay the formation of the complex.

It has been suggested[5] that ATP energetizes the muscle by converting the bond energy of its high-energy phosphate into an excitation energy on its purine ring, then transmitting this excitation energy to the contractile protein. How this transmission from the purine to the protein takes place is left open. The experiments with acridine orange suggest the possibility that, like the dye, the ATP molecules also form a complex with a constituent of muscle, probably a group on the protein—a complex capable of triplet excitation. This excitation of the ATP-protein dimer could represent the transmission of the energy of the nucleotide on to the protein.

The formation of complexes between ATP and acridine orange, on the one hand, and the formation of complexes between acridine orange and muscle, and between ATP and muscle, on the other hand, also suggest that dye and nucleotide might be bound by the same atomic groups of the contractile matter. If ATP and the dye react with the same atomic groups, then the dye should inhibit the contraction induced by ATP, and this inhibition should be a competitive one.

Experiments.—A thin bundle of glycerol-extracted psoas muscle fibers,[5] approximately 0.25 mm. in diameter, was cut in two in the middle. One half was allowed to contract in an ATP solution and served as a control for the other half, which contracted in a bath containing an identical ATP concentration but also containing acridine orange. This method eliminated errors due to variations among different fiber bundles. Contraction (shortening) was plotted against time. Acridine orange concentrations from 10^{-2} to 10^{-4} M were studied. By varying the ATP concentration, one set of experiments was performed for each acridine orange concentration employed. Since all experiments showed a uniform pattern, only a few representative examples will be described here.

Figure 1 illustrates the inhibitory effect of 10^{-2} M acridine orange on fibers contracting in a 2-, 4-, 8-, and 16-millimolar ATP solution. It can be seen that with 2 mM ATP the contraction is almost completely inhibited. Rising ATP concentrations diminish this inhibitory effect, and with 16 mM ATP, inhibition is minimal.

Similar experiments performed with lower acridine orange concentrations (10^{-3},

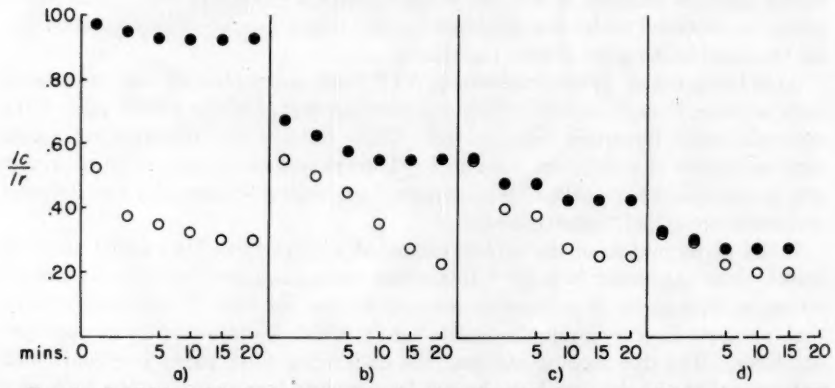


FIG. 1.—Inhibition of isotonic contraction by 10^{-2} M acridine orange. Effect of rising ATP concentrations: *a*, 2 mM ATP; *b*, 4 mM ATP; *c*, 8 mM ATP; *d*, 16 mM ATP. *Full circles:* fiber contracted in presence of 10^{-2} M acridine orange; *open circles:* fiber contracted in absence of acridine orange. l_r: rest length; l_c: contracted length.

5×10^{-4}, 10^{-4} M) showed only quantitative differences: a lesser degree of inhibition, which is further diminished with rising ATP concentration.

The inhibitory effect of a certain acridine orange concentration in the contraction bath was increased by incubating the fiber with the dye prior to contraction. Even short incubations of one minute and less were effective; longer incubations produced more pronounced inhibition. Rising ATP concentrations in the contraction bath diminished this additional inhibition. The pattern illustrated in Figure 1 was observed in all experiments.

With acridine orange concentrations as low as 10^{-4} M, in a contraction bath with 2 mM ATP, inhibitory effects were no longer shown. Even 20 minutes' incubation of the fiber in this dye concentration failed to produce an inhibitory effect for ATP concentrations of 2 mM and more (see Fig. 2, *a*). However, in the weaker and slower contractions obtained with 0.2 mM ATP, the inhibitory effect of 10^{-4} M acridine orange was again apparent (Fig. 2, *b*).

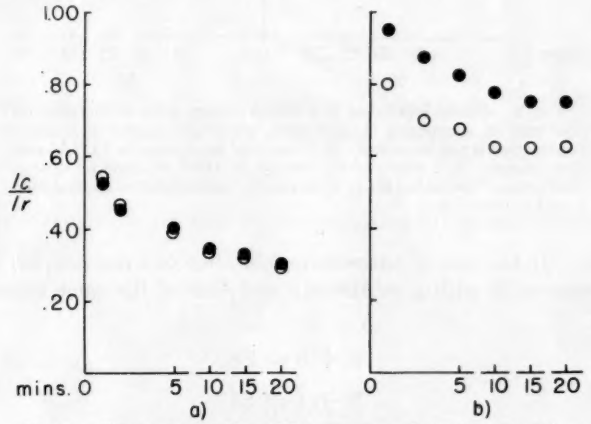


FIG. 2.—Effect of 10^{-4} M acridine orange on isotonic contraction: *a*, with 2 mM ATP; *b*, with 0.2 mM ATP. *Full circles:* fiber contracting in 10^{-4} M acridine orange after 20 minutes' incubation; *open circles:* control fiber in absence of acridine orange. l_r and l_c as in Fig. 1.

These opposite effects of ATP and acridine orange on the rate of shortening and degree of maximal contraction have been observed in all experiments. They seemed to suggest a competitive inhibition, i.e. a competitive adsorption of dye and ATP. Such a process would be analogous to the competitive adsorption of a substrate on its enzyme in the presence of a competitive inhibitor and should show characteristic quantitative relations. The formal and quantitative analysis of our experimental data should clarify this question and is presented below.

The reversibility of the acridine orange action could also be demonstrated by contracting acridine orange–incubated fibers in an ATP solution free of dyestuff. Here the rate of shortening was slowed, but finally the same maximal contraction was developed as by the nonincubated control fibers. Figure 3 illustrates two examples of these experiments. Maximal contraction was reached more rapidly with increasing ATP and more slowly with increasing dye concentration or pro-

longation of the time of incubation. This excludes a denaturation of the contractile proteins by the dyestuff under the experimental conditions employed.

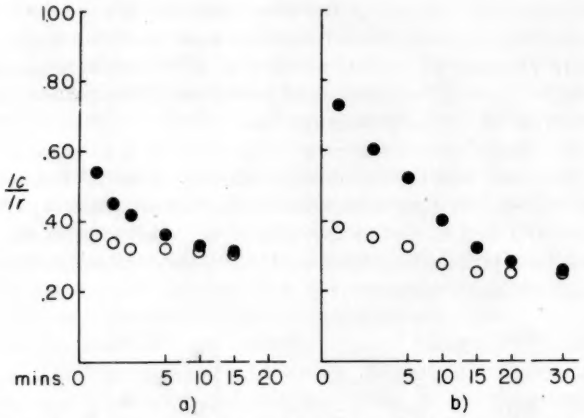


FIG. 3.—Fibers incubated in acridine orange prior to contraction. The rate of shortening is decreased, while the degree of maximal contraction is not impaired. *a*, 2 minutes' incubation in 10^{-3} *M* acridine orange; *b*, 2 minutes' incubation in 10^{-2} *M* acridine orange. *Full circles:* incubated fiber; *open circles:* nonincubated control fiber. l_r and l_c as in Fig. 1.

Discussion.—In the case of competitive inhibition of a reaction, as, for example, that of an enzyme E with a substrate S and that of the same enzyme with an inhibitor I,

$$E + S \rightleftarrows ES,$$

$$E + I \rightleftarrows EI,$$

ES and EI denote the enzyme-substrate and the enzyme-inhibitor complexes, respectively. The velocity v of the enzymic reaction is proportional to the concentration of ES. As is well known, if the reciprocal of the velocity of the enzymic reaction is plotted against the reciprocal of the substrate concentration, we obtain, in the case of competitive inhibition, a straight line which cuts the ordinate $(1/v)$ axis at a point which is independent of the inhibitor concentration, I. However, the slope is dependent on I. Consequently, the straight lines for different values of I cut the $(1/v)$-axis at the *same* point, whereas the slope is larger, the larger the value of I.

In the present case of muscle a constituent X corresponds to E, ATP to S, acridine orange (AO) to I. Thus

$$X + \text{ATP} \rightleftarrows X(\text{ATP}),$$

$$X + \text{AO} \rightleftarrows X(\text{AO}).$$

If we call K the equilibrium constant of the reaction of X and ATP, and K' that of the reaction of acridine orange and X, we have

$$\frac{C_0C_1}{C_2} = K \tag{1}$$

and

$$\frac{C_0C_3}{C_4} = K', \tag{2}$$

in which C_0 represents the concentration of the free X, C_1 that of free ATP, C_2 that of X(ATP), C_3 that of AO, and C_4 that of X(AO).

Furthermore, calling X_0 the total concentration of X, which is equal to the sum of that of the free X and that of the bound X in X(ATP) as well as in X(AO), we also have

$$C_0 + C_2 + C_4 = X_0. \tag{3}$$

Simple algebraic manipulation gives, from equation (1),

$$C_0 = K\frac{C_2}{C_1}, \tag{4}$$

and substitution of equation (4) into equation (2) leads to

$$C_4 = \frac{C_0C_3}{K'} = \frac{K}{K'}\frac{C_2C_3}{C_1}. \tag{5}$$

Introduction of equations (4) and (5) into equation (3) yields, after some simple rearrangements,

$$C_2\left(\frac{K}{C_1} + 1 + \frac{K}{K'}\frac{C_3}{C_1}\right) = X_0,$$

from which we obtain

$$C_2 = \frac{X_0}{1 + K/C_1 + (K/K')(C_3/C_1)},$$

or

$$C_2 = \frac{C_1}{C_1 + K(1 + C_3/K')}X_0. \tag{6}$$

Since, experimentally, the length of the muscle fiber was studied as function of time, the natural and most simple assumption which can be made here is that the shortening in the final (equilibrium) state is proportional to the concentration, C_2, of the X(ATP) complex:

$$l_r - l_c = \alpha C_2, \tag{7}$$

in which l_r is the rest length, l_c the length of the muscle fiber in the final (equilibrium) contracted state, and α a proportionality constant.

From equation (6) we see that the concentration C_2 is a (hyperbolically) increasing function of C_1 with saturation value X_0. Therefore, assumption (7) leads to

$$l_r - l_{mc} = \alpha X_0, \tag{8}$$

in which l_{mc} is the length of the maximally contracted fiber in its final (equilibrium) state, as occurs with a (infinitely) large concentration of ATP.

Elimination of α from equations (7) and (8) yields

$$\frac{l_r - l_{mc}}{l_r - l_c} = \frac{X_0}{C_2},$$

or

$$\frac{1}{l_r - l_c} = \frac{1}{l_r - l_{mc}} \frac{X_0}{C_2}. \tag{9}$$

Simple rearrangement from equation (6) leads to

$$\frac{X_0}{C_2} = 1 + K\left(1 + \frac{C_3}{K'}\right)\frac{1}{C_1}. \tag{10}$$

Introduction of expression (10) into equation (9) gives

$$\frac{1}{l_r - l_c} = \frac{1}{l_r - l_{mc}} + \frac{1}{l_r - l_{mc}} K\left(1 + \frac{C_3}{K'}\right)\frac{1}{C_1}, \tag{11}$$

from which we see that the intercept of the plot of the reciprocal of the shortening, $1/(l_r - l_c)$, versus that of the ATP concentration, $1/C_1$, should yield a straight line with an intercept equal to the reciprocal of the maximal shortening, $1/(l_r - l_{mc})$, which is *independent* of the inhibitor concentration C_3, and a slope $[1/(l_r - l_{mc})]$-$K(1 + C_3/K')$ which is a linear function of the inhibitor concentration C_3, as might have been expected from what has been said before about the competitive inhibition in general and assumption (7).

Without inhibitor, equation (11) leads, with $C_3 = 0$, to

$$\frac{1}{l_r - l_c} = \frac{1}{l_r - l_{mc}} + \frac{1}{l_r - l_{mc}} K\frac{1}{C_1}. \tag{12}$$

The plot of the value of $1/(l_r - l_c)$ versus $1/C_1$ for the experiment without acridine orange indeed yields a straight line, as shown in Figure 4, fitted by the least-squares method. From the intercept we obtain $1/(l_r - l_{mc}) = 1.14$, leading to $l_{mc} = 0.124$ (l_r is taken as unity), so that the maximal contraction with a very large concentration of ATP is 87.6 per cent. For the slope we find 7.37×10^{-4}, from which we obtain with the above-mentioned value of $1/(l_r - l_{mc})$ the value 6.5×10^{-4} for K.

Similarly, the plot of $1/(l_r - l_c)$ versus $1/C_1$, for an experiment containing acridine orange in the concentration 10^{-2} M, yields a straight line, as shown in Figure 4. For this line we find, by the method of least squares, the value 1.11 for the intercept $1/(l_r - l_{mc})$, which value is the same within the experimental accuracy as that found in the experiment without dye. The latter result proves that there is competitive inhibition of ATP and acridine orange for the same site of muscle constituent X.

Also from the slope 5.28×10^{-3} of the last straight line, from the above values of $1/(l_r - l_{mc})$ and K, and from the concentration $C_3 = 10^{-2}$ M of acridine orange, we find the value 15×10^{-4} for K', which shows that the dye is bound by the same site as ATP but is bound somewhat more than twice as strongly.

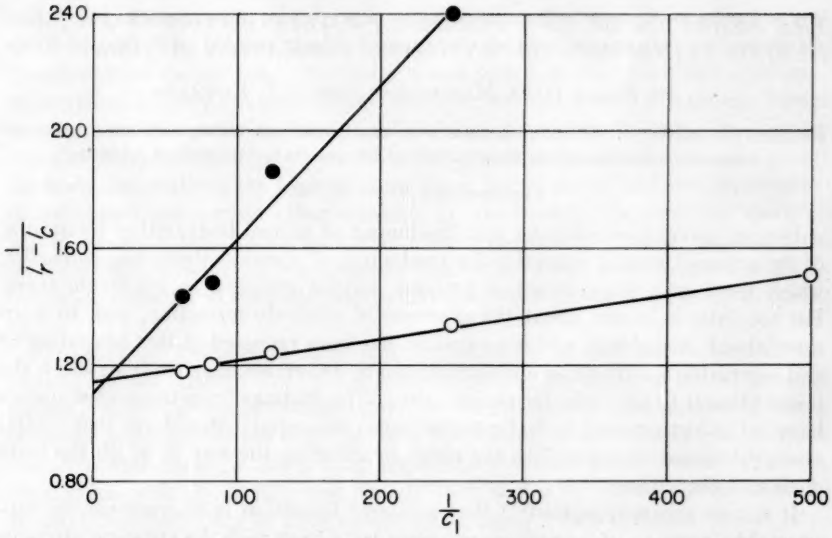


FIG. 4.—Reciprocal value of the final shortening plotted against the reciprocal value of the molar concentration of ATP. *Open circles:* fiber in absence of acridine orange; *full circles:* fiber in presence of 10^{-2} *M* acridine orange. l_c and l_r as in Fig. 1.

Summary.—Acridine orange and ATP form complexes with the same muscle constituents. The formation of the ATP complex is an essential step in the energetization of muscle. Acridine orange inhibits contraction, and this inhibition has the properties of a competitive absorption. Dye and ATP molecules compete for the same site.

* This research was sponsored by the National Institutes of Health, Grant No. H2042(R), the Commonwealth Fund, the American Heart Association, Inc., the Muscular Dystrophy Associations, the Association for the Aid of Crippled Children, the National Science Foundation, and the United Cerebral Palsy Foundation.

[1] A. Szent-Györgyi, *Bioenergetics* (New York: Academic Press, Inc., 1957).

[2] V. Zanker, *Z. physik. Chem.*, **199**, 225, 1952.

[3] E. Rabinowitch and L. F. Epstein, *J. Am. Chem. Soc.*, **63**, 69–78, 1941.

[4] A. Szent-Györgyi, *Biol. Bull.*, **96**, 140, 1949.

[5] A. Szent-Györgyi, *Henry Ford Hospital Symposium: Enzymes, Units of Biological Structure and Function*, ed. Oliver H. Gable (New York: Academic Press, Inc., 1955).

THE BEHAVIOR OF TWO FOREIGN PROTEIN ANTIGENS IN MICE DURING INHIBITION OF ANTIBODY FORMATION BY CORTISONE

By Philip D. McMaster and Joshua L. Edwards

ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH, NEW YORK, NEW YORK, AND DEPARTMENT OF PATHOLOGY, UNIVERSITY OF FLORIDA SCHOOL OF MEDICINE, GAINESVILLE, FLORIDA

Communicated March 18, 1957

Foreign proteins which enter into the human or animal body, either by natural or by artificial means, engender the production of special substances, *antibodies*, which react with these invading proteins, termed *antigens*, to inactivate them. Far too little is known about the processes of antibody formation, and, to learn more about the subject, a blue azoprotein has been prepared in this laboratory[1, 2] and injected into animals as a tracer antigen to determine the sites from which the initial stimuli to antibody formation arise. The findings from these experiments have led to further work to find out whether or not certain procedures that inhibit antibody formation accomplish the result by affecting the way in which the body deals with the antigen.

It is now generally agreed[3–7] that antibody formation in animals can be suppressed by cortisone if large doses are given prior to or with the antigenic stimulus and frequently thereafter. Indeed, under proper conditions, cortisone completely inhibits antibody formation in mice,[6, 7] an effect that is not attributable to rapid destruction of antibody as it forms.[4] What, then, can be learned by experiments employing tracer antigens in animals prevented from forming antibody by cortisone?

The first step in the formation of antibodies, as just mentioned, is the deposition of the antigen—whether it is an organism, a virus, or a protein—in phagocytic cells, usually those of the reticulo-endothelial system. Work from this laboratory[8, 9] and from many others[10] has shown that antibodies—although they may arise in various regions of the body—are formed to a large extent in lymph nodes and in the spleen. In these organs certain cells, macrophages and reticular cells which take up antigens and are believed by some to form antibodies, are far outnumbered by other nonphagocytic cells, such as lymphocytes and plasma cells, which, at the present time, are considered by the majority of workers to form or to carry antibodies.

Accordingly, the inhibition of antibody formation by cortisone might operate in several ways. For example, if the cells that take up antigen also form antibody, cortisone could affect either the capture of the antigen or the subsequent antibody formation. If the phagocytic cells, after taking up antigen, transfer it either intact or modified in some way to nonphagocytic cells, which subsequently form antibody, cortisone could also affect the transfer of the antigen or its modification, or the antibody-forming capacity of the non-phagocytic cells. Cortisone might act, too, by rapidly destroying the antigen or by breaking down the antibody as soon as it is formed, although the latter possibility seems to have been ruled out.[4]

LOCALIZATION OF A TRACER ANTIGEN IN MICE PREVENTED FROM FORMING ANTIBODIES BY THE ACTION OF CORTISONE

An excellent opportunity presented itself in the laboratory through the possession of the tracer antigen, mentioned earlier, to determine whether or not the inhibition

of antibody formation in mice by cortisone could be attributed to interference with the first step of the process, that is to say, with the phagocytosis or localization and distribution of the antigen. The tracer was obtained, as fully described elsewhere,[1] by coupling a highly diffusible, bright blue dye, *echt-säure-blau*, to a protein foreign to mice, namely, bovine γ-globulin. This blue dye protein, following its injection into the blood stream of mice, was taken up by reticulo-endothelial cells all through the body, especially in the liver and mesenteric lymph nodes, and to a lesser extent in the spleen and certain other organs.[1] It could readily be seen and identified within the cells either in sections or in cleared specimens. Before employing the tracer antigen, however, it was necessary first to find a way to obtain good antibody formation regularly in mice and next to find the proper dosage of cortisone to inhibit it.

Formation of Antibody in Mice and Its Inhibition by Cortisone.—Mice of two strains, one the outbred Rockefeller Institute strain, the other an inbred strain of Swiss mice, were given, at 48-hour intervals, two intraperitoneal injections of 10 mg. of bovine γ-globulin mixed in 0.9 per cent NaCl solution with *Hemophilus pertussis* vaccine containing eight billion killed organisms with each dose. Pertussis vaccine, when given with an antigen, had already been shown by others[11] to enhance the anaphylactic sensitivity of mice to various antigens. It was found in our laboratory to serve also as an adjuvant to antibody formation, so that mice injected with a mixture of bovine γ-globulin and the vaccine regularly produced good titers of circulating antibody detectable by the ordinary precipitin test. Next, hydrocortisone F was administered to mice so stimulated, by several routes, in different dosages and at various intervals, before and after giving the antigen-adjuvant mixture, until complete suppression of all antibody formation was regularly obtained for as long as the cortisone treatment was continued. Briefly, this was accomplished either by the subcutaneous implantation, 3 days before giving the antigen, of 3 mg. of hydrocortisone F in pellet form and the subsequent removal of the pellets when desired, or by intraperitoneal or subcutaneous injections of 2.5 mg. of the hormone 2 days before the first injection of the antigen. In all instances, whether or not pellets were used, injections of cortisone were given on the days that antigen was injected and also every 48 hours thereafter in doses of 1.5–2.5 mg.

Under these conditions the lymphoid organs showed extraordinary changes; the thymuses all but disappeared, and the spleens weighed about one-quarter, and the mesenteric lymph nodes one-fifth, or even one-ninth, as much as the same organs of normal mice of the same size as the experimental animals. The weights of the livers and kidneys remained relatively unchanged, although the animals lost about 20 per cent in total body weight. The changes were like those observed by Antopol[12] in mice given very large doses of cortisone.

Since the mice had received strong stimuli to antibody formation, it was necessary to maintain the high dosage of hydrocortisone mentioned above, in order to prevent the animals from forming antibody. Early in the work many of the mice succumbed to intercurrent infections, but it was soon found that the difficulty could be overcome by adding 50 mg. of terramycin to each liter of the animals' drinking water, beginning the use of the antibiotic a week before giving cortisone and continuing it throughout the experiment.

Tests with the Tracer Antigen.—Mice of both strains mentioned above were given cortisone, as just described, for 4 days, when 25 per cent of the animals, selected at

random, were killed and examined. The spleens, mesenteric nodes, and thymuses of all were greatly reduced in size. On the same day the remaining mice were injected intravenously with 1.0 mg. of the blue tracer antigen per 10 gm. of body weight, and the cortisone treatments were continued. An equal number of normal mice received similar injections of the tracer antigen but received no cortisone at any time. Three days later all were exsanguinated, and the spleens, mesenteric and peripheral lymph nodes, thymuses, livers, and kidneys were removed, weighed, and sectioned or were taken as whole organs to be cleared in methyl salicylate for examination.

Figure 1*a* shows, magnified 4 times, an unstained, cleared specimen of a typical mesenteric lymph node from one of the normal mice injected 3 days previously with the tracer. All the dark material in the photograph represents the sky-blue antigen situated in reticulo-endothelial cells in the node. Figure 1*b* indicates the appearance of a typical mesenteric node from one of the animals injected with the tracer while under the influence of cortisone. The shriveled node is deeply and intensely blue, almost black. This node contains far more of the antigen, per gram of tissue, than the node shown in Figure 1*a*. Figures 2*a* and 2*b* show, at the same magnification, cleared pieces of the spleens from the same animals. The lymph follicles which appear so prominently in the specimen from the normal mouse injected with the tracer (Fig. 2*a*) are practically lacking in the piece of the small spleen (Fig. 2*b*) from the mouse injected with the tracer while under the influence of cortisone. This organ is very deeply colored by its content of the blue tracer antigen. Photomicrographs (×264) of sections of mesenteric lymph nodes, like those shown in Figures 1*a* and 1*b*, cut to equal thickness and counterstained with neutral red, are shown in Figures 4*a* and 4*b*, respectively. The dark spots in each, which represent the blue antigen within phagocytic cells, illustrate how much more of the tracer is present, per unit of area, in the small node (Fig. 4*b*). The explanation is simple. Cortisone diminishes the size of the lymph nodes and spleens by reducing the number of lymphoid cells, while the reticulo-endothelial cells seem to

---

→

FIG. 1*a*.—The cleared, unstained, mesenteric lymph node from a normal mouse 3 days after an intravenous injection of the tracer antigen. ×4.

FIG. 1*b*.—The cleared mesenteric node from a mouse similarly injected while under the influence of cortisone. The dark areas in both photographs represent the blue tracer antigen. See text. ×4.

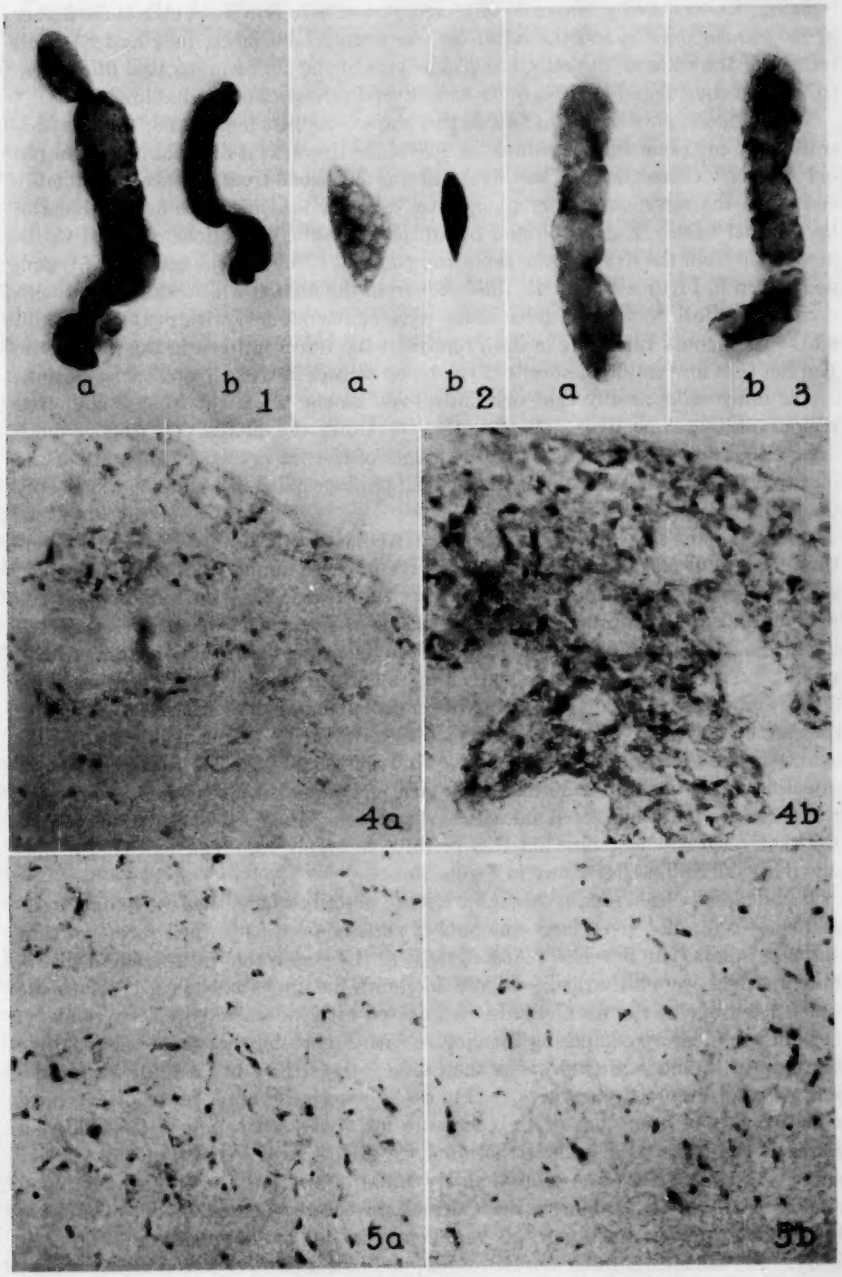
FIGS. 2*a* and 2*b*.—Cleared pieces of spleen from the same animals that furnished the nodes shown in Figs. 1*a* and 1*b*, respectively. ×4.

FIG. 3*a*.—The cleared mesenteric node from a normal mouse removed 6 weeks after injecting the blue antigen. ×4.

FIG. 3*b*.—The cleared mesenteric node from a mouse injected with the tracer antigen while the animal was under the influence of cortisone; the node must have been small, like that shown in Fig. 1*b*. Upon the withdrawal of cortisone, the node was allowed to regenerate for 6 weeks before it was removed. The organ contained about the same amount of the blue material as that shown in Fig. 3*a*. ×4.

FIGS. 4*a* and 4*b*.—Photomicrographs (×264) of sections of mesenteric nodes from mice treated like those which furnished the specimens shown in Figs. 1*a* and 1*b*, respectively. The tracer antigen appears as the dark material within the cells of the sinuses and the reticulum. The small node from the mouse treated with cortisone (Fig. 4*b*) contains much more antigen per gram of tissue than the node from the normal mouse (Fig. 4*a*). See text.

FIGS. 5*a* and 5*b*.—Sections of the livers (×264) from the mice which furnished the materials for Figs. 4*a* and 4*b*, respectively. Both livers contained approximately equal amounts of the tracer antigen, which appears as the dark specks in Kupffer cells and other endothelial cells. The liver of the cortisone-treated mouse (Fig. 5*b*) was about the same size as that of the normal mouse (Fig. 5*a*).


a
b 1
a
b 2
a
b 3
4a
4b
5a
5b

remain. Consequently, the ratio of phagocytic cells to lymphoid cells is far higher in the smaller organs, and the latter become deeply blue, since, judging by the intensity of the color in the cells, the phagocytes of the cortisone-treated mice seem to take up the colored antigen quite as well as do those of normal animals.

This supposition was put to test in two ways. As just mentioned, the administrations of cortisone did not reduce the size of the livers, as it did that of the lymphoid organs. Consequently, the livers of the cortisone-treated mice presumably contained the same number of phagocytic cells per unit of tissue as the livers of the normal mice. Figures 5*a* and 5*b* are photographs of sections of equal thickness made from the livers of the same animals that furnished the sections of lymph nodes seen in Figures 4*a* and 4*b*, the latter from the animal which was treated with cortisone. Both sections of liver tissue were lightly stained with neutral red, and all the dark spots appearing in both represent the tracer antigen in the phagocytic Kupffer cells and endothelial cells of the blood sinuses in these livers. The sections are as much alike as any that one finds, even among those cut from neighboring regions of the same liver. Apparently, therefore, the phagocytic cells of both organs took up approximately equal amounts of the tracer antigen, in spite of the fact that the animal furnishing the specimen photographed in Figure 5*b* was deeply under the influence of cortisone.

The point was shown in another way. Twenty test mice were treated with cortisone in the usual manner for 5 days. Five of these animals, killed at this time, showed greatly shriveled mesenteric nodes. On the same day the remaining fifteen mice and also eight normal animals of the same strain and weight were injected intravenously with equivalent amounts of the blue tracer antigen per gram of body weight. The cortisone injections given to the fifteen test mice were continued for 3 more days to maintain the reduced condition of the mesenteric lymph nodes during the period in which the tracer antigen would be taken up by them. By contrast, the eight normal mice received no cortisone. On the fourth day after injecting the antigen, five more of the fifteen test animals were bled and autopsied, together with half the normal animals. The nodes of the cortisone-treated animals were shriveled and deep blue like that pictured in Figure 1*b*. The nodes of the normal mice looked like that shown in Figure 1*a*.

It had already been found, through work not described here, that the lymph nodes and spleens of mice given large amounts of cortisone return to their former weight and size in less than 6 weeks. Accordingly, at the end of the sixth week all the remaining mice were autopsied. Figure 3*a* shows the appearance of a typical node taken from one of the normal animals injected with the tracer 6 weeks previously. It is to be noted, by comparing the picture with Figure 1*a*, that there was less blue material in the node after 6 weeks than after 3 days (Fig. 1*a*), a finding fully described and discussed elsewhere.[13] Figure 3*b* shows the appearance of a typical node which had been allowed to regenerate for 6 weeks within a mouse that had received the tracer while under the influence of cortisone. As Figures 3*a* and 3*b* show, the two nodes were approximately equal in size and apparently contained similar amounts of the blue antigen, which, in the regenerated node, must have been taken up while the organ was very small and the animal unable to form antibody. All the mice showed similar results, and so did those of a second experiment conducted in the same way.

Retention of the Antigenic Capacity by an Antigen Injected into Mice During the Inhibition of Antibody Formation by Cortisone.—Hayes and Dougherty[7] injected horse serum into cortisone-treated mice, continued the administration of cortisone for a few days, and then, several days after the cessation of treatment, found that antibodies to horse serum appeared. This technique was applied in the present work to learn whether or not the inhibition of antibody formation obtained by the large doses of cortisone used in the preceding experiments was produced by an effect upon the antigenicity of the antigen itself. To test the point, mice treated with cortisone for 3 days in the manner described were injected intraperitoneally on the fourth day with the antigen-adjuvant mixture of *H. pertussis* vaccine and 10 mg. of bovine γ-globulin. A second similar injection was given 48 hours later, while the cortisone treatment continued. As already mentioned, mice given similar injections but not treated with cortisone form readily detectable amounts of circulating, precipitating antibody. In a series of seven experiments, each employing 30–35 mice, the animals injected in this way continued to receive cortisone for 6, 8 (two experiments), 12, 16, 23, and 26 days, respectively, after the second injection of the mixture of antigen and pertussis vaccine. In each experiment several mice were bled at the termination of the cortisone treatments, and the presence of antibody was sought in their sera. In this report only experiments are included in which none of the animals formed any detectable antibody during the cortisone treatment. Four days after the withdrawal of the hormone, several mice in each experiment were bled, to test for evidence of antibody formation, and examined to determine the progress of the regeneration of the lymphoid and splenic tissues. At further intervals of 4 days similar groups from each experiment were bled and examined.

Findings.—When antibody formation was inhibited by cortisone for 6 and 8 days after the second injection of antigen, and the cortisone was then withdrawn, no antibody was found in the blood specimens obtained 4 days later, but it was present in those obtained on the eighth and twelfth days, respectively, after the withdrawal of the hormone, that is to say, on the fourteenth and twentieth days after the second injection of antigen. Antibody also appeared in the specimens taken after various intervals during the next 3 weeks. Upon the withdrawal of cortisone 12 days after the second injection of antigen—and antibody formation was inhibited during this period—no antibody was found in the sera obtained 4 and 8 days later, but it did appear in the specimens obtained on the twelfth day, 24 days after the second injection of antigen. In the three remaining experiments, in which cortisone administration was continued for 16, 23, and 26 days after the second injection of antigen, no antibody appeared at any time, although it was sought in surviving mice for 3 weeks.

DISCUSSION

The findings obtained in mice injected with the tracer antigen while they were profoundly under the influence of cortisone and unable to form antibody, and while the lymphoid and splenic tissues were very much smaller than in normal animals, indicate that the inhibition of antibody formation did not occur because the antigen was not taken up by the body. Nor does the suppression of antibody formation seem to be attributable to the rapid destruction of the antigen, since the experi-

ments described in the latter part of this paper show that a protein antigen injected into mice while cortisone is given to inhibit antibody formation can remain in the body under these circumstances for many days and still—when the cortisone is withdrawn—can stimulate antibody formation.

It may well be said that the antigen which was taken up by the animals persisted because no antibody was formed to destroy it. However this may be, other work from this laboratory,[14] to be reported elsewhere, throws some light on this question. In that work, rabbits, which form antibody well, were injected with one of the antigens used in the present experiments, bovine γ-globulin. While these animals, called "donors," were forming antibody, large amounts of liver tissue were taken and injected intraperitoneally into other rabbits, called "recipients." The recipients had previously been injected with the same antigen so that, upon a second contact with it, they would respond more readily with the formation of antibody. Liver tissue taken from the donors 2 weeks after they had received the antigen, when transferred to the recipients, stimulated the production of anti-bovine γ-globulin antibodies in the latter. Liver tissue taken from the donors 3 and 4 weeks after they had received the antigen failed to elicit antibody formation in the recipients. Obviously the antigen injected into the donor animals had survived for 2 weeks in a relatively intact form in spite of the fact that these rabbits formed antibody. Its survival in the mice of the present experiment for approximately the same time, in amounts sufficient to engender antibody formation later, indicated that its persistence was not due to the temporary lack of antibody formation.

SUMMARY

Mice given large amounts of hydrocortisone F fail to form antibodies to two foreign protein antigens which, as administered in the present work, effectively produce antibodies in normal animals. The distribution and storage of one of these antigens, a bright blue dye azoprotein derived from bovine γ-globulin, is much the same in mice during the inhibition of antibody formation by cortisone as it is in normal mice. The other antigen, bovine γ-globulin—injected into cortisone-treated mice unable to form antibody and prevented from producing it by further cortisone dosage for 12 days—remained in the body in a form capable of inducing the appearance of circulating antibody after another 12 days following the withdrawal of cortisone.

The mechanism by which cortisone inhibits antibody formation has, therefore, little if anything to do with the ability of the animals to take up or to store the protein antigens used in these experiments, nor does it seem to destroy the antigens more rapidly than usual. Accordingly, cortisone must influence either the subsequent utilization of the antigens or the actual formation of antibodies.

[1] H. Kruse and P. D. McMaster, *J. Exptl. Med.*, **90**, 425, 1949.

[2] P. D. McMaster, J. L. Edwards, and E. Sturm, *J. Exptl. Med.*, **102**, 119, 1955.

[3] F. G. Germuth, Jr., and B. Ottinger, *Proc. Soc. Exptl. Biol. Med.*, **74**, 815, 1950.

[4] F. G. Germuth, Jr., J. Oyama, and B. Ottinger, *J. Exptl. Med.*, **94**, 139, 1951.

[5] S. E. Newsom and M. Darrach, *Can. J. Biochem. and Physiol.*, **32**, 372, 1954.

[6] T. F. Dougherty, in *The Mechanism of Inflammation*, ed. G. Jasmin and A. Robert (Montreal: Acta, Inc., 1953).

[7] S. P. Hayes and T. F. Dougherty, *Federation Proc.*, **11**, 67, 1952.

[8] P. D. McMaster and S. S. Hudack, *J. Exptl. Med.*, **61**, 386, 1935.

[9] P. D. McMaster and J. G. Kidd, *J. Exptl. Med.*, **66**, 73, 1937.

[10] P. D. McMaster, "Sites of Antibody Formation," chap. 2 of *The Nature and Significance of the Antibody Response*, ed. A. M. Pappenheimer, Jr. (New York: Columbia University Press, 1953).

[11] S. Malkiel, B. J. Hargis, and S. M. Feinberg, *J. Immunol.*, **71**, 311, 1953.

[12] W. Antopol, *Proc. Soc. Exptl. Biol. Med.*, **73**, 262, 1950.

[13] P. D. McMaster and H. Kruse, *J. Exptl. Med.*, **94**, 323, 1951.

[14] P. D. McMaster and J. L. Edwards, **106**, 1857.

---

*PHOTO SPIN RESONANCE IN CHLOROPHYLL-CONTAINING PLANT MATERIAL**

BY POWER B. SOGO, NING G. PON, AND MELVIN CALVIN

RADIATION LABORATORY AND DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY

Communicated by Melvin Calvin, March 8, 1957

Introduction.—In a recent paper,[1] the electron spin resonance spectra obtained from light-induced paramagnetism in chloroplast suspensions were described, and a discussion of the possible role of these unpaired electrons in the processes of photosynthesis was given. We have verified the essential features of the results reported above and have made new measurements in an attempt to more closely establish the origin of the signal. The characteristic features of the resonance absorption are the g value, the growth and decay times, the steady-state concentration of the absorbing centers, the quantum efficiency for the production of the absorbing centers, and the shape of the spin resonance spectrum. The determination of the identity of the absorbing species, as well as the path of its formation, would seem to require, first, a knowledge of the precise dependence of the above quantities on such variables as temperature, light wave length and intensity, sample preparation, and sample environment, and, second, the correlation of such data with the results of chemical and kinetic studies of photosynthesis. It is hoped that in this way a fundamental insight into the primary processes of energy conversion in green plants will be gained. This paper describes a series of exploratory experiments in which the primary aim was the determination of the relative importance of several variables in affecting the behavior of the signal—in particular, temperature, light intensity, and sample preparation.

Methods.—In these experiments a total weight of 250 gm. of spinach leaves was required for the preparation of whole chloroplasts and smaller fragments. The leaves were washed with distilled water, and the excess water was shaken off. All subsequent operations were carried out at, or near, 0° C.

In a typical experiment, 125 gm. of fresh spinach leaves was sliced into thin strips and ground for 30 seconds in a blender containing 250 ml. of 0.5 M sucrose, pH 6.8, and 0.03 M with respect to potassium phosphate. The homogenate was filtered through 6 layers of cheese cloth. The filtrate was centrifuged at 200 $\times$ g for 5 minutes to remove whole cells and cell debris. The supernatant was carefully decanted and centrifuged at 600 $\times$ g for 12 minutes. The supernatant was decanted and saved for the preparation of the small chloroplast fragments, while the

precipitate was resuspended in *ca.* 200 ml. of fresh sucrose solution. A rubber policeman attached to one end of a glass rod was used to obtain an even suspension. The suspension was centrifuged again at 600 × *g* for 15 minutes. The supernatant was discarded, and the precipitate was collected by resuspension in a small volume of fresh sucrose solution and centrifugation at 20,000 × *g* for 5 minutes. The supernatant was quickly decanted, and the packed precipitate so obtained was designated "whole chloroplasts."

One-half of the "whole chloroplasts" precipitate was suspended in *ca.* 10 ml. of pH 6.8, 0.05 *M* phosphate buffer, and the suspension was allowed to stand overnight at 0° C. The mixture was centrifuged at 10,000 × *g* for 10 minutes. The supernatant was discarded, while the precipitate was washed once again with phosphate buffer and collected by centrifugation at 10,000 × *g* for 10 minutes. Again the supernatant was discarded, and the tightly packed precipitate was designated "large chloroplast fragments."

For the preparation of the small chloroplast fragments, the 600 × *g* supernatant was centrifuged for 1 minute at 20,000 × *g* to remove any remaining whole chloroplasts. The light green supernatant was decanted and centrifuged at 20,000 × *g* for 15 minutes. The supernatant was quickly decanted. The loosely packed green precipitate was designated "small chloroplast fragments." The latter probably contain, in addition to small fragments of chloroplasts, mitochondrial particles. A flow diagram of the preparations is given in Figure 1.

All the above preparations were kept at 0° C. until ready for spin resonance studies. The spin resonance spectra were obtained with a transmission-cavity spectrometer operating at a frequency of about 9.3 kMc/sec, with a bolometer for the detector.[2] Magnetic field modulation at a frequency of 165 cps and a conventional lock-in amplifier are employed for the display of the derivative of the absorption on the chart recorder. Frequency stabilization of the Varian V-58 klystron on the peak of maximum cavity transmission is effected with a frequency-modulation feedback system.

The variable temperature probe is shown in Figure 2. The samples were painted on the surface of a $^5/_{32}$-inch O.D. silver-plated copper rod that was inserted into a clear quartz Dewar. The completed assembly was then placed in the cavity resonator. This change in cavity geometry converts the TE_{011} mode right cylinder into a TE_{011} coaxial cylinder with a small decrease in cavity *Q*. However, the resulting favorable sample shape, with a large surface-area-to-volume ratio, together with the small value of the electric field throughout the sample volume, more than compensates for the disadvantage of the drop in *Q*. The sample temperature was changed by immersing the bottom end of the rod in a suitable bath, and the resulting temperature was monitored by a thermocouple attached to the rod in the vicinity of the sample.

The light source consisted of a 200-watt tungsten-filament projection lamp. A Corning No. 3480 sharp-cut filter in conjunction with a special water-cooled Corning infrared filter served to restrict the band width of the incident light to the wave lengths lying between 5800 and 8000 A. A pair of condensing lenses was used to collect and focus the light on the 1-cm. hole at the end of the wave-guide tee. After transversing the length of the tee, the light entered the cavity through a 6-mm. iris.

Results.—A summary of the results is given in Table 1. Eucalyptus leaves showed a faint light-dependent signal when they were air-dried to a proper state of

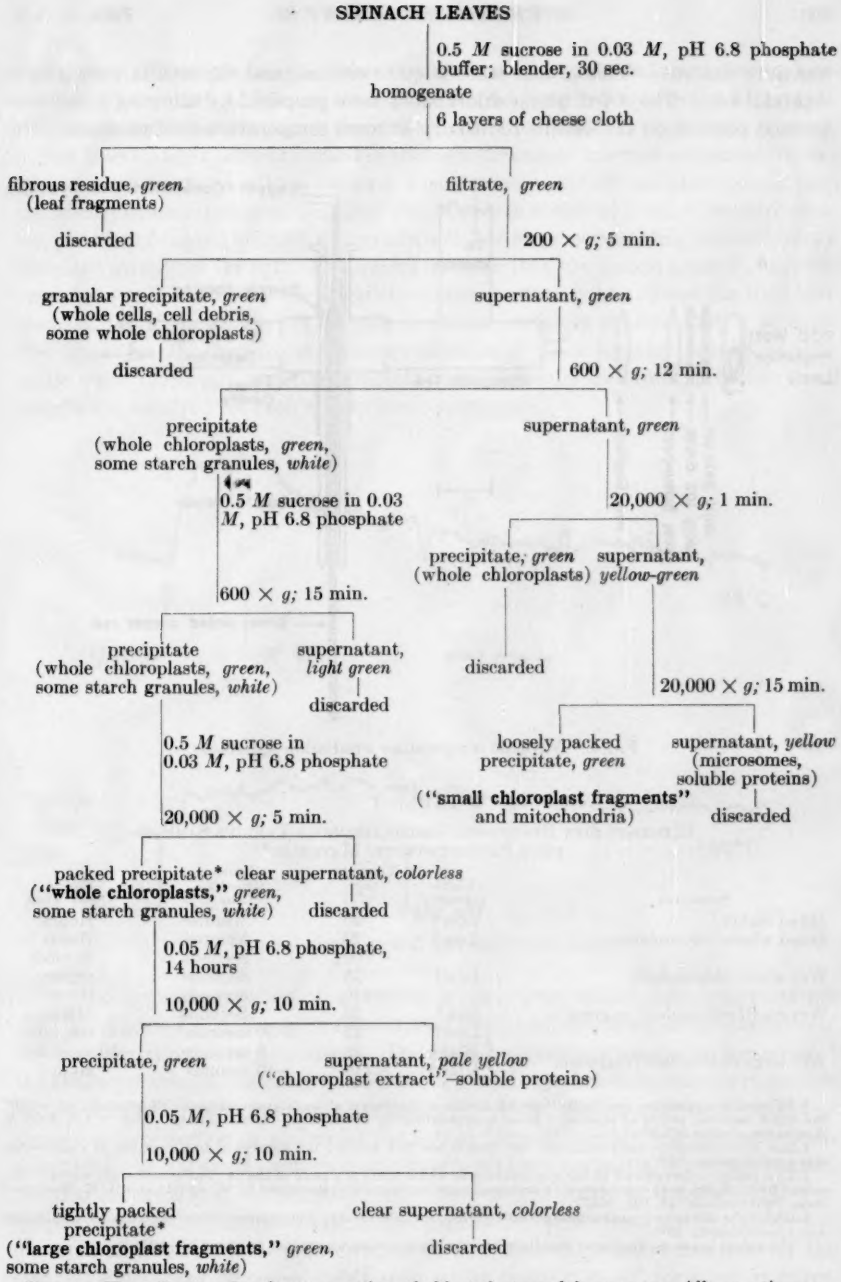

SPINACH LEAVES
0.5 M sucrose in 0.03 M, pH 6.8 phosphate buffer; blender, 30 sec.
homogenate
6 layers of cheese cloth
fibrous residue, green (leaf fragments)
discarded
filtrate, green
200 × g; 5 min.
granular precipitate, green (whole cells, cell debris, some whole chloroplasts)
discarded
supernatant, green
600 × g; 12 min.
precipitate (whole chloroplasts, green, some starch granules, white)
0.5 M sucrose in 0.03 M, pH 6.8 phosphate
600 × g; 15 min.
supernatant, green
20,000 × g; 1 min.
precipitate, green (whole chloroplasts)
supernatant, yellow-green
discarded
20,000 × g; 15 min.
precipitate (whole chloroplasts, green, some starch granules, white)
supernatant, light green
discarded
0.5 M sucrose in 0.03 M, pH 6.8 phosphate
20,000 × g; 5 min.
loosely packed precipitate, green ("small chloroplast fragments" and mitochondria)
supernatant, yellow (microsomes, soluble proteins)
discarded
packed precipitate* ("whole chloroplasts," green, some starch granules, white)
clear supernatant, colorless
discarded
0.05 M, pH 6.8 phosphate, 14 hours
10,000 × g; 10 min.
precipitate, green
supernatant, pale yellow ("chloroplast extract"–soluble proteins)
0.05 M, pH 6.8 phosphate
10,000 × g; 10 min.
tightly packed precipitate* ("large chloroplast fragments," green, some starch granules, white)
clear supernatant, colorless
discarded

FIG. 1.—Flow diagram for the preparation of chloroplasts and fragments. All operations are carried out at, or near, 0° C.

* Samples were taken by skimming material from the top; white starch material was at the very bottom of the centrifuge tube.

moisture content. This proper state is quite critical, and the results were poorly reproducible. The dried whole chloroplasts were prepared by allowing a wet suspension painted on the sample rod to dry at room temperature for two days. The

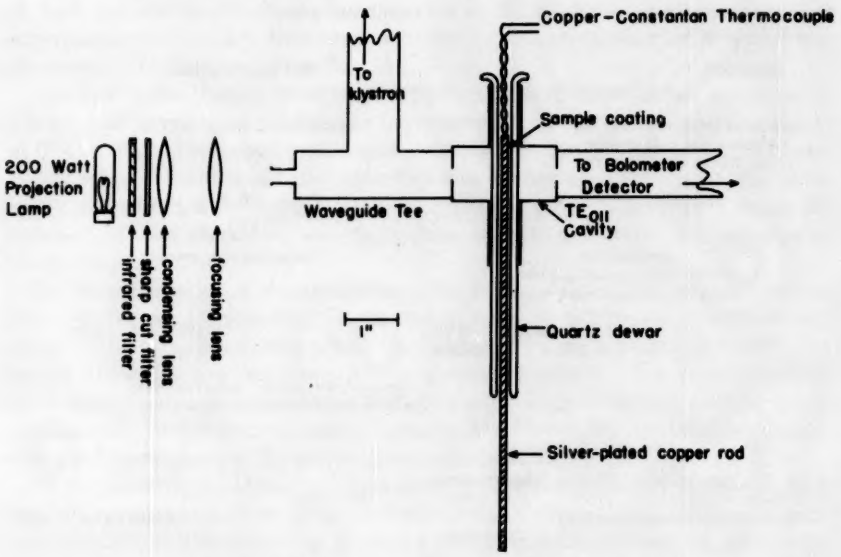


FIG. 2.—Variable temperature irradiation apparatus

TABLE 1

ELECTRON SPIN RESONANCE OBSERVATIONS ON VARIOUS SAMPLES FROM PHOTOSYNTHETIC MATERIAL*

Substance	Light Intensity	Temperature (° C.)	Signal Growth Time	Signal Decay Time
Dried leaves	Low†	25	Minutes	Hours
Dried whole chloroplasts	Low†	25	Minutes	Hours
		60‡	Seconds	Seconds
Wet whole chloroplasts	Low†	25	Seconds	~1 minute
		−140	Seconds	Hours
Wet small chloroplast fragments	Low†	25	Seconds	Minutes
Wet large chloroplast fragments	Low†	25	~30 seconds	~30 seconds
	High§	25	~6 seconds	~30 seconds
	High§	−140	~10 seconds	Hours‖

* At room temperature the width between points of maximum slope is approximately 10 oersteds; at −140° the width between points of maximum slope is approximately 15 oersteds. Light quality: $5800 < \lambda < 8000$ A. Resonance g value is 2.00.

† Low light intensity: approximately 10^{15} quanta/sec into cavity; the number of free electrons at equilibrium was approximately 10^{16}.

‡ This seems to correspond to the temperature at which there is a peak in the thermoluminescence curve. Observed by G. Tollin in this laboratory in an experiment similar to that described by W. Arnold and H. K. Sherwood these PROCEEDINGS, *43*, 105, 1957.

§ High light intensity: approximately 10^{18} quanta/sec into cavity; the number of free electrons at equilibrium was approximately 10^{16}.

‖ The signal began to disappear rapidly when the temperature was raised to about $> -50°$.

signal growth and decay times for the wet, large chloroplast fragments were obtained by setting the magnetic field on a peak of maximum slope and recording the change in this peak height as a function of time. Representative traces are shown

in Figure 3. The instrumental time constant was set at 2 seconds in these measurements. The growth and decay times for the other samples were obtained by repetitive scanning of the resonance absorption, and their order of magnitude is given.

The filtered light intensity passing through the cavity iris was estimated for two optical configurations with a General Electric type DW-60 radiation meter, and the steady-state number of unpaired electrons was obtained from a comparison of the product of (signal height) $\times$ (signal width)^2 to the corresponding product from a standard sample of DPPH. The results indicate that the fastest growth times obtained are still limited by the intensity of the incident light. Thus the time constant of the signal-producing process is almost certainly shorter than 6 seconds. The quantum efficiency for the photoproduction of the absorption centers appears to be quite high; although the absolute measurements are crude, probably 1–10 quanta are required for each absorption center.

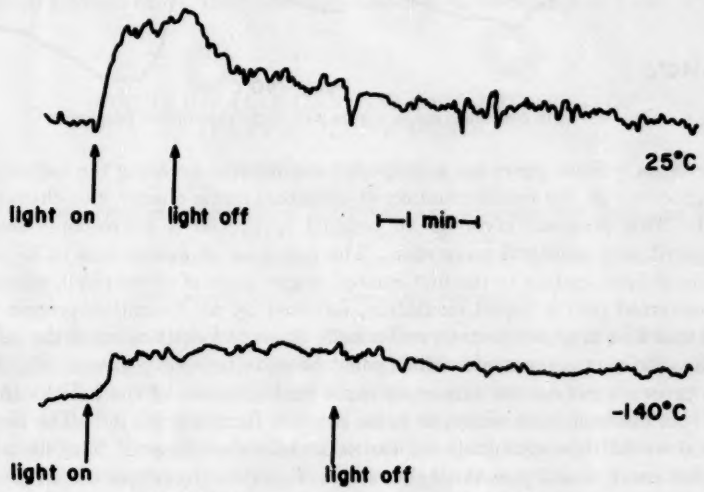


FIG. 3.—Signal-growth and decay-time curves

Typical resonance curves of a sample of wet, large chloroplast fragments are shown in Figure 4. A change in the shape of the light-induced signal as a function of the sample temperature is evident. The room-temperature resonance has some of the characteristics of exchange narrowed lines, while the low-temperature resonance is considerably broader and more asymmetric. This behavior may be the effect of anisotropic crystalline or molecular fields that interact weakly via spin-orbit coupling with the unpaired electron in the frozen solid and are partially averaged out by thermal motions at room temperature. The residual signal in the dark differs in shape from the light-induced signal, and it probably arises from a different species of unpaired electrons. It is interesting to note that the relative intensity of the residual signal may be considerably reduced by heating at 40° for a few minutes. No apparent destruction of the samples is caused by the rapid cooling to $-140°$. When such frozen samples are allowed to warm up to room temperature, their behavior is the same as that of samples kept continuously at room temperature.

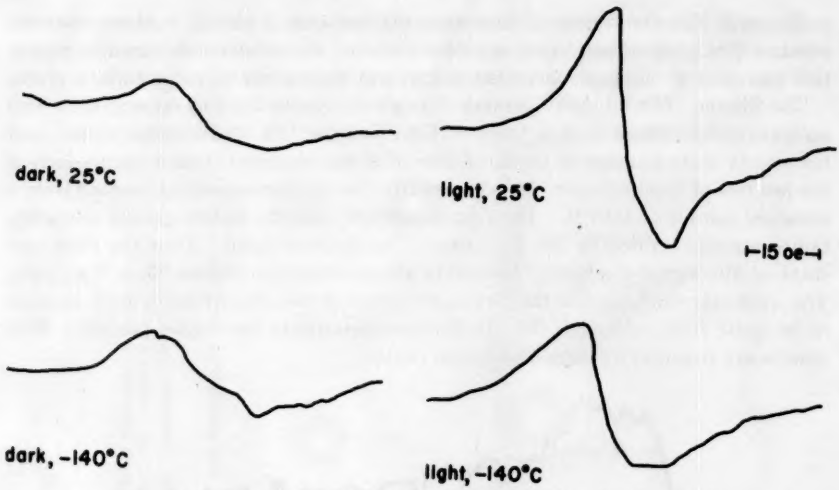


FIG. 4.—Spin resonance spectra from wet, large chloroplast fragments

Discussion.—Some years ago a proposal[3] was made concerning the nature of the early processes in the transformation of electromagnetic energy into chemical potential. This proposal involved an ordered array, on a microscopic scale, of chlorophyll and collateral molecules. The sequence of events was to be an absorption of light leading to the first excited singlet state of chlorophyll, which then was converted into a triplet excitation, followed by an ionization process which would lead to a trapped electron and a hole, these two entities being the reducing and the oxidizing components which must be simultaneously generated. A part of the process involved the extremely rapid neutralization of the hole by the capture of an electron from water, or some product formed from it.[4] The resultant material would then ultimately appear as a molecular oxygen. The electron, on the other hand, would pass through a series of carriers (hydrogen carriers) such as are well known in biochemical processes (thioctic acid, pyridine nucleotide, flavin, etc.), ultimately leading to the reduction of carbon dioxide.

According to this scheme, one should expect at least three seats of electronic paramagnetism to occur, namely, the triplet state of chlorophyll, the trapped unpaired electrons, and the radicals of the semiquinone type among the hydrogen carriers on the path between the trapped electron and carbon dioxide. Within experimental error, the signal growth time for frozen, wet, large chloroplast fragments at $-140°$ appears to be the same as the growth time for the corresponding fluid sample at 25°. This fact seems sufficient for ruling out the ordinary enzymatic reactions as intermediates in the signal-producing process. Thus, according to the above scheme, we are left with the chlorophyll triplet, the trapped electron, and, additionally, some species of free radical resulting from the direct dissociation of a chemical bond in the absorption act. While it is possible to suppose that the cooling would enhance the lifetime of the chlorophyll triplet to the extent of hours, it does not seem likely. We are thus left with the trapped electron and the possibility of a dissociated bond.

It is perhaps worth noting that whatever the nature of the unpaired electron producing this signal, its coupling with the lattice around it must be rather poor in order to produce a signal as narrow as the one we see, suggesting its location in a rather delocalized pi-type of orbital. It is to be expected that improvements in technique will lead to a more precise identification of the variety of unpaired electrons which almost certainly result from the illumination of the photochemical apparatus in plants.

* The work described in this paper was sponsored by the United States Atomic Energy Commission.

[1] B. Commoner, J. J. Heise, and J. Townsend, these PROCEEDINGS, **42**, 710, 1956.

[2] R. Beringer and J. G. Castle, *Phys. Rev.*, **78**, 581, 1950.

[3] D. F. Bradley and M. Calvin, these PROCEEDINGS, **41**, 563, 1955.

[4] A very similar concept of the semiconductor qualities of the chloroplast was proposed on the basis of fluorescence studies quite independently by E. Katz, *Photosynthesis in Plants*, ed. W. E. Loomis and J. Franck (Ames, Iowa: Iowa State College Press, 1949), chap. 15, p. 291.

---

*BIOCHEMICAL BASIS FOR THE TOXICITY OF PENTACHLOROPHENOL**

BY EUGENE C. WEINBACH

UNITED STATES DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE, PUBLIC HEALTH SERVICE, NATIONAL INSTITUTES OF HEALTH
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES,† BETHESDA, MARYLAND

Communicated by Charles Armstrong, February 25, 1957

Although drugs and other toxic substances have been used by man for centuries, it is only in recent years that any clear insight into their mechanism of action has been gained. Current biochemical knowledge and techniques have provided powerful tools for the study of drug action at the cellular and subcellular levels. Such a study of the mechanism of action of pentachlorophenol has been undertaken in the Laboratory of Tropical Diseases. Pentachlorophenol has been used in the field as a molluscicide to control schistosomiasis since 1949. It is also used industrially to suppress the growth of many types of undesirable micro-organisms, and its toxicity to vertebrates is well documented. Since pentachlorophenol is toxic to a wide range of living organisms, either it is nonspecific in action, affecting a large number of unrelated metabolic processes, or it affects a basic mechanism common to many forms of life. For reasons which will be developed here, it appears that the latter concept is the more likely one.

Since our original interest in pentachlorophenol was concerned with its molluscicidal properties, it seemed desirable to study first the effect of this compound on the over-all metabolism of living snails.

When living specimens of *Australorbis glabratus* (the intermediate molluscan host of the schistosomes of man in the Western Hemisphere) are exposed to minute concentrations of pentachlorophenol, the drug accumulates and concentrates in their tissues. The tissue concentration reaches a level of 2.5×10^{-4} M after 24 hours' exposure to pentachlorophenol at 7.5×10^{-6} M (2 parts per million).[1] Paralleling the accumulation of pentachlorophenol in the snail tissues is a pro-

nounced change in their metabolism. This is seen by analysis of tissues removed from snails after their exposure to the chlorophenol. Pyruvate, acetate, lactate, and orthophosphate levels in the snails exposed to pentachlorophenol increased over the levels found in the nonexposed or control snails.[1] The very large increase in acetate and lactate under the influence of pentachlorophenol is remarkably similar to the normal end products of anaerobic metabolism (glycolysis) of these snails.[2] Another reflection of their altered metabolism is the increased levels of pyruvate and phosphate. This effect is reversible, for, if the snails are removed from pentachlorophenol after only a few hours' exposure, their metabolism returns essentially to normal.[1]

An additional effect of *low* concentrations of pentachlorophenol upon intact, living snails is to increase their respiration. This stimulating action of pentachlorophenol takes several hours to reveal itself—presumably owing to the time required for the drug to penetrate and/or concentrate in their tissues. Higher concentrations of the chlorophenol cause a marked depression in the respiratory rate. Similar effects were noted with isolated snail tissues.[1] Relevant to this discussion is the observation that pentachlorophenol stimulates the respiration of higher laboratory animals also.[3]

The major finding that emerged from our experiments with living snails was that pentachlorophenol stimulates glycolysis and accelerates respiration. These effects resembled the action of 2,4-dinitrophenol with other organisms.[4] It is well established that dinitrophenol increases the metabolic rate of higher organisms, including man. Furthermore, the early work of Loomis and Lipmann demonstrated that dinitrophenol prevented the formation of energy-rich phosphate compounds which accompanied the oxidation of carbohydrate by cell-free rabbit kidney preparations.[5] Since pentachlorophenol and dinitrophenol are structurally related compounds, one might anticipate that they share similar physiological properties. Indeed, it seemed likely that interruption of aerobic phosphorylation might be the basis of pentachlorophenol's toxicity. However, as is well known but sometimes forgotten, reasoning by analogy alone can be erroneous. For example, trinitrophenol, while closely related structurally to dinitrophenol, has absolutely no effect on phosphorylation in the usual concentration ranges. Thus it was necessary to study experimentally the effect of pentachlorophenol on phosphorylating enzymes.

Data are presented in Table 1 which illustrate the effect of pentachlorophenol upon oxidative phosphorylation in rat liver mitochondria. In the control experiment, approximately 15 micromoles of inorganic phosphate were taken up for

TABLE 1

EFFECT OF PENTACHLOROPHENOL ON OXIDATIVE PHOSPHORYLATION (RAT LIVER MITOCHONDRIA)*

	PO_4 UPTAKE (MICROMOLES)		AA FORMED (MICROMOLES)		P:O	
MOLARITY	PCP	DNP	PCP	DNP	PCP	DNP
0 (control)	15.4	15.4	5.3	5.3	2.9	2.9
5×10^{-4}	0	0	3.1	3.3	...	...
5×10^{-5}	0	0	5.0	5.1	...	...
5×10^{-6}	4.0	12.9	5.3	5.3	0.8	2.4
5×10^{-7}	13.6	15.5	5.2	5.3	2.6	2.9

* Oxidative phosphorylation was carried out as described by E. C. Weinbach and J. Garbus, *Nature*, *178*, 1225–1226, 1956.

every 5 micromoles of acetoacetic acid formed. The micromoles of acetoacetate (AA) formed are stoichiometrically equivalent to the microatoms of oxygen consumed during the oxidation of β-hydroxybutyrate. Thus a P:O ratio approaching the maximum theoretical value of 3 was obtained. In the presence of minute concentrations of pentachlorophenol (PCP), phosphate uptake was suppressed completely or "uncoupled." For comparative purposes, data obtained with 2,4-dinitrophenol (DNP) are included. At the highest concentration listed, 5×10^{-4} *M*, both compounds also partially impeded the oxidation. However, at 5×10^{-5} *M*, there was complete inhibition of phosphate uptake, with no significant interference with substrate oxidation. It is at lower concentrations that the effectiveness of PCP as an uncoupler is readily demonstrated. At 5×10^{-6} *M*, pentachlorophenol exerted strong uncoupling, while dinitrophenol was only weakly effective.[7] At the lowest concentration employed, 5×10^{-7} *M*, both compounds were practically inactive, although even at this extremely low concentration PCP still exhibited feeble uncoupling properties. Similarly, we have demonstrated that pentachlorophenol also dissociates the phosphorylation coupled to the oxidation of α-ketoglutaric acid.[8]

Data presented elsewhere reveal that pentachlorophenol is a powerful uncoupler of oxidative phosphorylation in snail as well as in rat tissues.[9] Again it was observed that oxidation of the substrate (β-hydroxybutyrate) was not impaired by low concentrations of PCP, while the coupled phosphorylation reaction was completely blocked. An important point to be emphasized here is that the same concentration range of PCP that completely uncouples oxidative phosphorylation in the in vitro system is found in the tissues of snails moribund from exposure to the halophenol (2.5×10^{-4} *M*).

These observations suggested that pentachlorophenol, by denying aerobic exergonic reactions to the organism, caused increased glycolytic activity, as reflected by the increase in lactate and acetate discussed earlier. This may be looked upon as a compensatory mechanism, since glycolysis also leads to net adenosine triphosphate (ATP) synthesis. Such a hypothesis implies that oxidative but not glycolytic phosphorylative reactions are interrupted by PCP in snail tissues. Thus it was necessary to examine the effects of pentachlorophenol on the molluscan enzymes involved in glycolytic phosphorylation.

The results of such experiments revealed that pentachlorophenol in the same concentration range that completely uncouples oxidative phosphorylation, and the same range found in snail tissues after exposure to lethal doses of PCP, has no effect on glycolytic phosphorylation in the in vitro system.[9] Similar effects were found with mammalian enzymes.

Pentachlorophenol has other biochemical properties which may be related to its toxicity. A characteristic of most uncoupling agents is their ability to accelerate ATP breakdown in the presence of mitochondria. However, pentachlorophenol, in contrast to dinitrophenol, exerts a dual effect upon the mitochondrial ATPase obtained from either molluscan or mammalian tissues.[9, 10] In experiments with rat liver mitochondria whose structure had been destroyed either by acetone or by exposure to sonic oscillation, low concentrations of either dinitrophenol or pentachlorophenol increased the amount of phosphate released from added ATP.[10] However, as the concentrations of the substituted phenols were increased, DNP

continued to stimulate ATPase, while PCP was strongly inhibitory. Recently we have obtained similar results with the important physiological phosphatase, myosin ATPase (Table 2). These experiments suggest that PCP may hinder intracellular transfer of energy-rich phosphates as well as their synthesis.

TABLE 2

EFFECT OF PCP AND DNP ON MYOSIN ATPASE*

Molarity	PCP	DNP
0	15.9	15.9
5×10^{-5}	17.6	15.0
5×10^{-4}	19.7	16.8
1×10^{-3}	4.5	17.5
5×10^{-3}	0.2	18.4

* Values are expressed as micromoles of phosphate released from 24 micromoles of ATP in 10 minutes at 25°. Details of the assay are given by S. V. Perry, in S. P. Colowick and N. O. Kaplan (eds.), *Methods in Enzymology* (New York: Academic Press, 1955), 2, 582. Myosin B was a gift from Dr. William J. Bowen.

Another unusual biochemical property of pentachlorophenol is its effect on the morphology of mitochondria in vitro. When isolated mammalian or molluscan mitochondria are treated with rather high concentrations of PCP (5×10^{-3} *M*), there is rapid swelling and subsequent dissolution of these particulates. Dinitrophenol, on the other hand, does not exhibit this effect. Although these gross effects are not observed at lower concentrations of pentachlorophenol, there is some loss of mitochondrial nucleotides and other nitrogenous material into the medium.[10] Since it has been generally observed that alteration of mitochondrial structure rapidly inactivates aerobic phosphorylations, such structural changes may also play a role in the toxicity of pentachlorophenol, especially in the higher concentration range. However, it should be realized that as yet no observations concerning the possible occurrence of such changes in vivo are available.

As summarized in Table 3, consideration of all the experiments described suggests the following sequence of events concerning the toxicity of pentachlorophenol:

TABLE 3

PENTACHLOROPHENOL TOXICITY

Concentration of PCP	In Vitro Effect	Possible Physiological Significance
10^{-6}–10^{-4} *M*	Uncoupling of oxidative phosphorylation	Interference with cellular aerobic exergonic processes
10^{-4}–10^{-3} *M*	Inhibition of mitochondrial ATPase	?
	Inhibition of myosin ATPase	Interference with phosphate transfer (and muscle function?)
10^{-3} *M* and higher	Inhibition of glycolytic phosphorylation	Rapid death of the cell and of the organism
	Inactivation of respiratory enzymes	
	Gross damage to mitochondrial structure	

In low concentrations (10^{-6}–10^{-4} *M*), oxidative phosphorylations are interrupted. This interferes with the aerobic cell's primary exergonic or energy-yielding mechanism. Since glycolytic phosphorylations are not inhibited by these low concentrations, the organism may survive for a limited time through utilization of glycolytic exergonic reactions. However, there is little doubt that uncoupling of

oxidative phosphorylation in itself could ultimately prove fatal for an aerobic organism.[4] Furthermore, since oxidative phosphorylation is a common biochemical denominator among a large variety of living organisms, interruption of this mechanism could account for pentachlorophenol's wide range of toxicity. At higher concentrations, 10^{-4}–10^{-3} M, mitochondrial and myosin ATPase are inhibited. Although the physiological role of mitochondrial ATPase is obscure, the inhibition of myosin ATPase suggests interference with phosphate transfer and possibly muscle function. At 10^{-3} M and higher, glycolytic as well as oxidative enzymes are completely suppressed, with rapidly fatal results. At these higher concentrations, changes in morphology of the particulate elements of the cell also may occur, as suggested by the gross damage to mitochondrial structure in the in vitro experiments.

In any consideration of the mode of drug action, it must be borne in mind that several mechanisms may be in operation at once and that it is unlikely that any compound is so selective as to react with only one enzyme or enzyme system. Thus, yet to be elucidated is the mechanism by which pentachlorophenol exerts its lethality on certain organisms under anaerobiosis, where aerobic exergonic reactions are nonexistent. This must remain a problem for the future.

* Presented at the autumn meeting of the National Academy of Sciences, November 8, 1956, Washington, D.C.

† Laboratory of Tropical Diseases.

[1] E. C. Weinbach and M. O. Nolan, "The Effect of Pentachlorophenol on the Metabolism of the Snail *Australorbis glabratus*," *Exptl. Parasitol.*, **5**, 276–284, 1956.

[2] T. von Brand, P. McMahon, and M. O. Nolan, "Observations on the Post-anaerobic Metabolism of Some Fresh-Water Snails," *Physiol. Zoöl.*, **28**, 35–40, 1955.

[3] W. Deichmann, W. Machle, K. V. Kitzmiller, and G. Thomas, "Acute and Chronic Effects of Pentachlorophenol and Sodium Pentachlorophenate upon Experimental Animals," *J. Pharmacol. Exptl. Therap.*, **76**, 104–117, 1942.

[4] E. W. Simon, "Mechanisms of Dinitrophenol Toxicity," *Biol. Revs. Cambridge Phil. Soc.*, **28**, 453–479, 1953.

[5] W. F. Loomis and F. Lipmann, "Reversible Inhibition of the Coupling between Phosphorylation and Oxidation," *J. Biol. Chem.*, **173**, 807–808, 1948.

[6] E. C. Weinbach and J. Garbus, "Age and Oxidative Phosphorylation in Rat Liver and Brain," *Nature*, **178**, 1225–1226, 1956.

[7] Perhaps related to this observation is the finding that pentachlorophenol is approximately ten times more effective as a molluscicide than is dinitrophenol.

[8] E. C. Weinbach, "The Effect of Pentachlorophenol on Oxidative Phosphorylation," *J. Biol. Chem.*, **210**, 545–550, 1954.

[9] E. C. Weinbach, "The Influence of Pentachlorophenol on Oxidative and Glycolytic Phosphorylation in Snail Tissue," *Arch. Biochem. and Biophys.*, **64**, 129–143, 1956.

[10] E. C. Weinbach, "Pentachlorophenol and Mitochondrial Adenosinetriphosphatase," *J. Biol. Chem.*, **221**, 609–618, 1956.

*EVIDENCE FOR "GIBBERELLIN-LIKE" SUBSTANCES FROM FLOWERING PLANTS**

By Bernard O. Phinney, Charles A. West, Mary Ritzel, and Peter M. Neely

DEPARTMENT OF BOTANY AND DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, LOS ANGELES

Communicated by G. W. Beadle, March 8, 1957

Gibberellins are compounds that produce a wide variety of growth responses in flowering plants. Among these responses are stimulation of shoot growth for many plant species,[1] root elongation in maize,[2] induction of bolting and flowering in some biennial and long-day plants,[3–5] resumption of normal growth in certain genetically dwarfed genotypes of maize,[6] growth stimulation in dwarf strains of peas and beans,[7] and the overcoming of physiological dwarfing of apple seedlings.[8] Gibberellins also stimulate parthenocarpy in tomatoes,[9] promote the germination of light-requiring lettuce seed in the dark,[10] and reverse the light inhibition of pea stem growth.[11]

For the interpretation of these and other effects, it is important to know whether gibberellins or "gibberellin-like" substances occur naturally in flowering plants. The three known gibberellins, gibberellic acid (gibberellin A_3), $C_{19}H_{22}O_6$; gibberellin A (gibberellin A_1), $C_{19}H_{24}O_6$; and gibberellin A_2, $C_{19}H_{26}O_6$, are products of certain strains of the imperfect stage of the fungus *Gibberella fujikuroi* (Saw.) Wr.[12–16] No other organism has been found to contain these compounds.

The present paper will report evidence for the occurrence of "gibberellin-like" substances from species of several different families of flowering plants.[17] These substances produce the same growth response in maize dwarfs as do the known gibberellins. R_f values for these substances will also be reported and compared with those for the known gibberellins.

MATERIALS AND METHODS

Ten genetically different dwarf mutants of *Zea mays* L. were used in this study, Nine of them are simple recessives, while one is a simple dominant. Gibberellic acid, gibberellin A_1, and gibberellin A_2 produce normal growth for the five mutants *anther ear-1*, *dwarf-(5232)*, *dwarf-1*, *dwarf-2*, and *dwarf-3*, the qualitative response to each of the three gibberellins being similar in the seedling stages. Four other mutants, *dwarf-(4963)*, *dwarf-(8043)*, *nana-1*, and *nana-2*, show only a very slight response in the seedling stages and little response or slight inhibition in later stages of growth. The other mutant, *dominant dwarf*, gives no response to any of the three gibberellins.

All ten mutants were used to compare the biological specificity of response of the known gibberellins with the "gibberellin-like" substances obtained from flowering plants (Angiosperms). The mutant *dwarf-1* was used for routine bioassays because of its specificity, sensitivity, and rapidity of response. This mutant does not respond in the bioassay to indole-3-acetic acid, indoleacetonitrile, indoleacetic acid ethyl ester, naphthaleneacetic acid, naphthoxyacetic acid, 2,4-dinitrophenoxyacetic acid, kinetin, leucoanthocyanins from *Aesculus* endosperm and *Arachis* seed, casein hydrolyzate, yeast extract, malt extract, or coconut milk. The mutant gives a positive response to 0.001 μg. of gibberellic acid per plant and a detectable response within 8 to 12 hours following application of 0.1 μg. of gibberellic acid per plant.

Material for bioassay was applied as a small drop to the first unfolding seedling leaf at the time of its emergence from the coleoptile. For semiquantitative work, the volume added for assay was arbitrarily standardized at a single application of 0.1 ml. per plant. For qualitative tests, larger volumes were often used, applications being repeated over a period of 24 hours. All assays were run in triplicate. Following treatment, the seedling was allowed to grow until the first leaf sheath had reached its final length, a period of from three to five days following application. Record of response was obtained by measuring to the nearest millimeter the length of this leaf sheath from the ligule to the coleoptilar node. Increases in length ranged from 0 to 400 per cent over that of the largest nontreated control plant (*dwarf-1*). Only those increases greater than 25 per cent were considered as evidence for activity in screening experiments. The sensitivity of the bioassay was increased by adding the detergent "Tween-20" (sorbitan polyoxyethylene monolaurate) at the level of two drops per 100 ml. of the aqueous solution to be tested. "Tween-20" gave no response by itself. Materials showing no activity were tested several times in aqueous solutions as well as in solutions of acetone-water (1:1) and ethyl alcohol–water (1:1).

The following procedures were typical for the preparation of extracts and diffusates from higher plants for bioassay. Shelled market peas, fresh weight 250 gm., were shaken for 24 hours in 300 ml. of acetone-water (1:1). The solvent was filtered and an aliquot of the filtrate evaporated to dryness on a steam cone. The oily residue was resuspended in a small volume of water containing "Tween-20" and the suspension assayed for activity. Extracts were obtained from maize by grinding kernels in the milk stage in a Waring Blendor with acetone-water (1:1). The resulting slurry was shaken for 48 hours and the extract processed as described above. In certain cases the endosperm dissected from young seed was applied directly to the test plants.

For paper-partition chromatography, the active extracts or diffusates were applied as a narrow band through a 10-cm. width of 14 × 43-cm. Whatman No. 1 filter paper. A spot of gibberellic acid (5 μg.) was applied as a reference compound in the remaining 4-cm. zone. The chromatogram was then developed by descending solvent flow in a closed tank at room temperature. After the solvent had migrated to a point 30 cm. from the origin, the chromatogram was dried, and the 4-cm. zone containing the gibberellic acid was cut from the chromatogram. The 10-cm. strip was then cut into 2-cm. sections at right angles to the direction of the solvent flow, and the 15 sections thus obtained were extracted separately with a total of 5 ml. of acetone-water (1:1). The eluants were evaporated to dryness and the residues dissolved or suspended in a minimum amount of water containing "Tween-20." The position of the active zone for the plant extract was determined by bioassay, that of the gibberellic acid control by a fluorescence test (see below). R_f values were calculated from these data.

The fluorescence test described by Cross[12] was adapted for the detection of gibberellic acid on the chromatograms. The chromatograms to be tested were soaked with concentrated sulfuric acid and exposed to ultraviolet light. Under these conditions, as little as 0.1 μg. of gibberellic acid in a spot 1 cm. in diameter gave a characteristic blue fluorescence. Gibberellin A_1 and gibberellin A_2 did not give a detectable response to this test. Ehrlich's aldehyde reagent[18] was used as a spray

test for the detection of indole derivatives on the chromatograms. Vanillin reagent[19] was used as a spray test for the detection of leucoanthocyanins and similar compounds.

PRESENTATION OF DATA

Screening of Plant Materials for Activity in the Bioassay.—Methanol extracts from maize kernels in the milk stage were active in the bioassay, as were extracts and diffusates from the seed or fruit of a number of species of flowering plants. The endosperm from *Echinocystis*, *Aesculus*, and *Persea* gave evidence of activity when tested directly. The activities of the various plant sources are given in Table 1. The response of *dwarf-1* seedlings to *Echinocystis* endosperm and to diffusates of *Phaseolus* are shown in Figures 1 and 2.

TABLE 1

"GIBBERELLIN-LIKE" ACTIVITY FROM EXTRACTS OR DIFFUSATES OF FLOWERING PLANTS

(Activity Is Evidenced by a Growth Response in *dwarf-1* Seedlings)

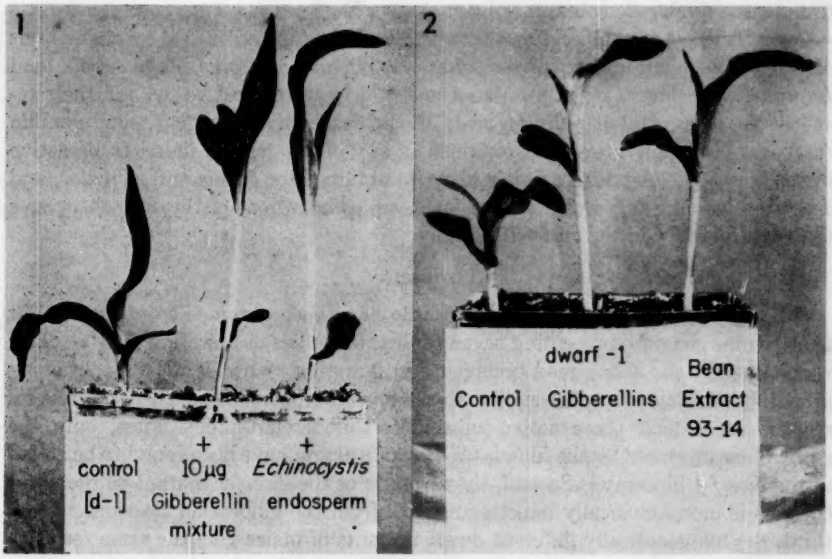
Plant Source	Part	Extraction Solvent	Activity
Phaseolus vulgaris L. (bean)	Green seed, from small to full size	Ethyl ether	+
		Acetone-water	+
		Acetone	+
Pisum sativum L. (pea)	Green seed, from small to full size	Acetone-water	+
		Ethyl ether	+
Aesculus californica (Spach.) Nutt. (California buckeye)	Endosperm	*	+
Echinocystis macrocarpa Greene (wild cucumber)	Endosperm	*	+
Lupinus spp. (lupine)	Green seed, full size	Ethyl ether	+
Zea mays L. (Maize)	Fruit, milk stage	Ethyl ether	+
		Methanol	+
		Acetone	±
Persea americana Mill. (avocado)	Endosperm	*	+
Prunus domestica L. (plum)	Endosperm, young seed	Ethyl ether	+
Prunus armeniaca L. (apricot)	Endosperm, young seed	Ethyl ether	+
Prunus amygdalus Batsch. (almond)	Endosperm, young seed	Ethyl ether	+
Datura stramonium L. (Jimson weed)	Young fruit	Ethyl ether	−
Juglans regia L. (English walnut)	Endosperm, young seed	Ethyl ether	−
Juglans californica Wats. (California walnut)	Endosperm, young seed	Ethyl ether	−
Nicotiana glauca Graham (tree tobacco)	Young fruit	Ethyl ether	+

* Liquid endosperm tested directly.

Specificity of Response of the Ten Dwarf Mutants to the Plant Extracts.—Extracts or diffusates from *Echinocystis*, *Aesculus*, *Persea*, *Lupinus*, *Phaseolus*, and *Pisum* were tested with each of the ten dwarf mutants to see whether the specificity of response followed that of the gibberellin response. It was found that these extracts were active for the five mutants that responded to the gibberellins, slightly active for the four mutants that showed a slight response to the gibberellins, and inactive for the mutant, *dominant dwarf*, that showed no response to the gibberellins.

Chromatographic Comparison of Active Plant Extracts with the Gibberellins.—Extracts or diffusates prepared from *Phaseolus*, *Pisum*, *Aesculus*, *Echinocystis*, and *Lupinus*, and were compared with each other and with the known gibberellins by

means of paper-partition chromatography. The results from four different solvent systems are shown in Table 2. The three known gibberellins could not be distinguished from each other on the basis of R_f values in any of the solvent systems used.



FIGS. 1 AND 2.—Response of *dwarf-1* seedlings to gibberellins and to the "gibberellin-like" substances from *Echinocystis* (wild cucumber) and from *Phaseolus* (bean). *From left to right: dwarf-1* nontreated control, *dwarf-1* treated with a mixture of gibberellic acid and gibberellin A_1, and *dwarf-1* treated with plant extract. Plant extract in Fig. 1 is the endosperm of one half-developed *Echinocystis* seed. Response is confined to the second leaf sheath, because of the age of the assay plants. Plant extract in Fig. 2 is a diffusate from young bean seed.

TABLE 2

CHROMATOGRAPHIC COMPARISON OF ACTIVE PLANT EXTRACTS AND THE GIBBERELLINS*

MATERIAL TESTED	SOLVENT SYSTEM A	B	C	D
Gibberellic acid	0.25	0.58	0.85	0.74
Gibberellin A_1	0.24	0.55	0.89	0.72
Gibberellin A_2	0.29	0.61	0.87	0.73
Phaseolus (bean)	0.27	0.65	0.85	0.76
Pisum (pea)	0.43	0.65	0.91	0.73
Aesculus (buckeye)	0.43	0.66	0.86	0.74
Echinocystis (wild cucumber)	0.51	0.77	0.87	...
Lupinus (lupine)	0.11	0.48	0.67	...

* R_f values are the average of two or more determinations which agreed within ±5 per cent. Solvent systems consisted of the upper phase of an immiscible system prepared by mixing 3 volumes of *n*-butanol and 1 volume of 1.5 *N* ammonium hydroxide for solvent A; 35 volumes of *n*-amyl alcohol, 35 volumes of pyridine, and 30 volumes of water for solvent B; and 95 volumes of *n*-butanol, 5 volumes of glacial acetic acid, and 30 volumes of water for solvent C. Solvent D consisted of 8 volumes of ethanol and 2 volumes of 3 *N* ammonium hydroxide.

Bean extracts gave R_f values similar to the gibberellins in three of the solvent systems but gave a consistently higher R_f value in solvent system B. When bean extract and gibberellic acid were co-chromatographed in this latter system, an active zone was found in front of the gibberellic acid zone. The active materials from

Pisum and *Aesculus* were similar to each other in migratory properties but were different from those of *Phaseolus* and the gibberellins. The active components of *Echinocystis* and *Lupinus* gave migratory properties differing from each other and from the other active components tested, including the gibberellins. All materials tested gave similar R_f values in solvent system D.

Color Tests of the Active Plant Extracts and the Gibberellins.—Chromatograms of the active plant extracts from *Phaseolus, Pisum, Aesculus, Echinocystis,* and *Lupinus,* were developed in *n*-butanol–acetic acid–water and tested for their response to concentrated sulfuric acid, Ehrlich's aldehyde reagent, and vanillin reagent. No positive test was obtained in any of the regions found to be active in the bioassay. A positive indole test was obtained for *Pisum* and *Aesculus,* and a positive vanillin test was obtained for *Aesculus* in regions of the chromatograms that were inactive in the *dwarf-1* bioassay.

DISCUSSION

Substances active in the *dwarf-1* bioassay have been obtained from the seed or fruit of nine genera representing seven different families of flowering plants. This would suggest the widespread occurrence in flowering plants of substances having growth-promoting properties similar to the known gibberellins. There are several reasons for calling these active substances "gibberellin-like." First, only the gibberellins produced by the fungus *Gibberella fujikuroi* have been found to be active in the *dwarf-1* bioassay. Second, the response of the *dwarf-1* mutant to the plant extracts is morphologically indistinguishable from the gibberellin response. And, third, the ten genetically different dwarf mutants of maize give the same response to the plant extracts as to the gibberellins. That is, the plant extracts are active for the five mutants that respond to the gibberellins, and inactive or only slightly active for the five mutants that do not respond or respond only slightly to the gibberellins.

The negative test from extracts from four other species of flowering plants could be due to the conditions of extraction and testing or could mean that such substances may be absent from the seed or fruit of these species or present only in minute amounts.

The evidence for the chemical relationship of the "gibberellin-like" substances to the known gibberellins is indirect. The regions of the chromatograms active in the bioassay give no color tests for indole compounds and for leucoanthocyanins, nor do they fluoresce in sulfuric acid as does gibberellic acid. Indole compounds, leucoanthocyanins, kinetin, and sources known to be rich in growth factors are not active in the bioassay. Finally, R_f values of the plant extracts are similar in some but not all of the solvent systems tested. In fact, the R_f values from the plant extracts vary depending on the source, suggesting that there may be a number of "gibberellin-like" substances in flowering plants.

Recently, Radley[20] has reported evidence for substances like gibberellic acid obtained from purified extracts of young pea shoots. On paper chromatograms, using chloroform: ethanol: water: formic acid (20:4:2:1), an active zone was found having the same R_f value as gibberellic acid. Radley[20] has suggested that the phytohormone reported by Mitchell *et al.*[21] in 1951 was probably a gibberellin. Mitchell had found that ether diffusates from young bean seed would give an 879 per cent in-

crease in length for the first internode, with additional growth stimulation of the internodes above this region. Indole-3-acetic acid also gave a 310 per cent increase with this test, but the IAA response was confined to the first internode of the bean seedlings. The unknown phytohormone was best obtained from very young seed. Activity disappeared by the time the seed were relatively mature. Our *dwarf-1* bioassay shows the presence of a "gibberellin-like" substance in bean seed from shortly after pollination to *green* seed that have reached full size. Mature green seed of other species were also found to be active.

Our data demonstrate the widespread occurrence in flowering plants of substances having properties similar to those of the gibberellins. These "gibberellin-like" substances must be isolated and purified before the precise chemical relation to the known gibberellins can be established.

SUMMARY

1. Extracts which give a "gibberellin-like" response in the *dwarf-1* bioassay have been obtained from nine genera representing seven different families of flowering plants.

2. Five genetically different dwarf mutants of maize respond both to the gibberellins produced by the fungus *Gibberella fujikuroi* and to the "gibberellin-like" substances obtained from the seed or fruit of flowering plants. Four other dwarf mutants respond only slightly in the seedling stage to the gibberellins and to the "gibberellin like" substances. Another mutant, *dominant-dwarf*, gives no growth response to the gibberellins or to the "gibberellin-like" substances.

3. The morphological response of the mutants to the "gibberellin-like" substances is indistinguishable from the response to the known gibberellins.

4. A comparison of R_f values for the "gibberellin-like" substances from six plant species and for the known gibberellins suggests that (*a*) these "gibberellin-like" substances are not *chemically identical* with the known gibberellins and (*b*) there may be a number of "gibberellin-like" substances present in flowering plants.

* This work was supported in part by Research Corporation. The authors wish to thank Dr. E. G. Anderson, of the California Institute of Technology, for the use of certain information on the genetics of the dwarf mutants reported here. Samples of gibberellic acid and gibberellin A were furnished by Dr. Curt Leben, of Eli Lilly and Company; Dr. Reed Gray, of Merck and Company, Incorporated; and Dr. Frank H. Stodola, of the United States Department of Agriculture, Peoria, Illinois. A sample of gibberellin A_2 was kindly provided by Dr. Y. Sumiki, of Tokyo University, Japan.

[1] B. B. Stowe and T. Yamaki, *Ann. Rev. Plant Physiol.*, **8**, 1957 (in press).

[2] W. G. Whaley and J. Kephart, *Science*, **125**, 234, 1957.

[3] A. Lang, *Naturwissenschaften*, **43**, 544, 1956.

[4] F. Lona, *Nuovo giorn. botan. ital.*, N.S., **63**, 61–76, 1956.

[5] R. Bunsow and R. Harder, *Naturwissenschaften*, **43**, 527, 1956.

[6] B. O. Phinney, these PROCEEDINGS, **42**, 185–189, 1956.

[7] P. W. Brian and H. G. Hemming, *Physiol. Plantarum*, **8**, 669–681, 1955.

[8] L. V. Barton, *Contribs., Boyce Thompson Inst.*, **18**, 311–317, 1956.

[9] S. H. Wittwer, M. J. Bukovac, H. M. Sell, and L. E. Weller, *Plant Physiol.*, **32**, 39–41, 1957.

[10] A. Kahn, J. A. Goss, and D. E. Smith, *Science*, **125**, 645–646, 1957.

[11] J. Lockhart, these PROCEEDINGS, **42**, 841–848, 1956.

[12] T. Yabuta and T. Hayashi, *J. Agr. Chem. Soc. Japan*, **15**, 257–266, 1939.

[13] B. E. Cross, *J. Chem. Soc.*, pp. 4670–4676, 1954.

[14] F. H. Stodola, G. E. N. Nelson, and D. J. Spence, *Arch. Biochem. and Biophys.*, **66**, 438–443, 1957.
[15] A. Kawarada, H. Kitamura, Y. Seta, N. Takahashi, M. Takai, S. Tamura, and Y. Sumiki, *J. Agr. Chem. Soc. Japan*, **19**, 278–281, 1955.
[16] N. Takahashi, H. Kitamura, A. Kawarada, Y. Seta, M. Takai, S. Tamura, and Y. Sumiki, *Bull. Agr. Chem. Soc. Japan*, **19**, 267–277, 1955.
[17] C. A. West and B. O. Phinney, *Plant Physiol.*, **31** (suppl.), xx (abstr.), 1956.
[18] F. Feigl, *Spot Tests*, Vol. II: *Organic Applications* (New York: Elsevier Publishing, Co., 1954), p. 199.
[19] E. C. Bate-Smith and N. H. Lerner, *Biochem. J.*, **58**, 126–132, 1954.
[20] M. Radley, *Nature*, **178**, 1070–1071, 1956.
[21] J. W. Mitchell, D. P. Skaggs, and W. P. Anderson, *Science*, **114**, 159–161, 1951.

---

*THE EFFECT OF HETEROZYGOSITY FOR NEW MUTATIONS ON VIABILITY IN DROSOPHILA: A PRELIMINARY REPORT**

By Bruce Wallace

BIOLOGICAL LABORATORY, COLD SPRING HARBOR, NEW YORK

Communicated by Th. Dobzhansky, March 16, 1957

Geneticists are nearly unanimous in their belief that the vast majority of mutations are deleterious. This belief rests primarily upon the effect of mutations in the homozygous condition. The standard with which these homozygous individuals are compared is generally provided by individuals heterozygous for the mutations under investigation; the relatively excellent viability of these latter individuals is ascribed to the dominance of normal, "wild-type" alleles.

There exists in the literature a sizable record of well-documented instances of heterozygotes being superior in viability or fitness to both homozygotes; many of these are the cases of balanced polymorphism observed in natural populations. Some observations suggest that heterosis can be produced in individual instances by mutations with a marked deleterious effect when homozygous as well as an average deleterious effect in the heterozygous condition. Thus, Wallace and King,[1, 2] Cordeiro,[3, 4] and Dobzhansky *et al.*[5] have studied the relative viabilities of individuals homozygous and heterozygous for chromosomes, including lethal chromosomes, from experimental and natural populations and have found ample evidence for the occurrence of heterotic chromosomal combinations. Stern *et al.*,[6] in reporting on the average decrease in viability of *Drosophila* females heterozygous for sex-linked lethals, call attention to two lethals among the seventy-seven tested which appear heterotic; an analysis of these data by a technique reported by Wallace and Madden[7] suggests that the proportion of heterotic lethals among those tested may even exceed 20 per cent. Morton, Crow, and Muller[8] refer to another study of the viability of individuals heterozygous for "recessive" lethals. They point out that the variance between viabilities of individuals heterozygous for different lethals in this study is not significantly greater than that between replicate tests of the same lethal; the observed variances (F-ratio of 1.76) differ by an amount, however, which indicates that these data, also, are not inconsistent with the occurrence of a sizable proportion of heterotic lethals.

Convincing evidence for heterosis must of necessity rest on well-documented instances in which the heterozygote differs widely from the two homozygotes, and, as long as our attention is concentrated solely upon cases of this sort, heterosis will be considered a rare, and in some respects unimportant, phenomenon in the genetics of populations. It would be a heroic task, if not a physical impossibility, to proceed experimentally, locus by locus, obtaining data on the occurrence of heterotic mutations. For one thing, the estimated number of loci is prohibitively large; second, it is possible that only a small proportion of gene changes at any one locus can be recognized as such. The experiments described below, using *D. melanogaster*, endeavor to avoid this difficulty. Techniques are known by which the viability of individuals homozygous for a given chromosome can be determined. The question asked by this study can be stated in the following way: What is the average effect of a random array of new mutations on the viability of such homozygotes when each mutation (1) is present in the heterozygous condition, and (2) represents the only change in the original homozygous genotype? A full account of these experiments will be published elsewhere; the present report outlines the experimental technique and summarizes the results obtained until now.

From the routine tests of our experimental population No. 18 (see Wallace[9]), four chromosomes were obtained, each of which, when homozygous, gave viable and fertile adults. These chromosomes were introduced singly into the genetic background of a *CyL/Pm* stock (*Cy*, *L*, and *Pm* are the dominant genes Curly wing, Lobe eye, and Plum eye; the *CyL* chromosome also has inversions that suppress crossing over) by repeated (eleven or more) backcrosses of single *CyL/+* males with *CyL/Pm* females of the stock culture. An experiment involving one of these chromosomes was initiated by crossing *CyL/+* brothers and sisters of one of these backcross cultures and by collecting the +/+ male offspring of this mating. These males were divided into two groups, one of which was exposed to approximately 500 r X-radiation. (Irradiated chromosomes will be indicated by ⊕.) The irradiated males were mated singly to *CyL/Pm* virgin females, single *Pm/*⊕ sons were remated to *CyL/Pm* females, and three *Pm/*⊕ males were obtained from each of these F_2 cultures. Simultaneously, the nonirradiated +/+ males were mated with *CyL/Pm* females, single *Pm/+* sons were remated with *CyL/Pm* females, and three *Pm/+* males and eight *CyL/+* virgin females were obtained from each F_2 culture. Four of the *CyL/+* females from each culture were mated with the three *Pm/*⊕ males from the irradiated series which happened to carry the same identifying number. The other four *CyL/+* females of each nonirradiated culture were mated with the three *Pm/+* males of a *different* culture of the nonirradiated series. Avoiding brother-sister matings in these latter crosses insured that the relationship between the parents in the two series of matings was the same.

The expected results of the matings described above are as follows:

Control	1 *CyL/Pm* : 1 *CyL/+* : *Pm/+* : 1 +/+
X-ray	1 *CyL/Pm* : 1 *CyL/*⊕ : 1 *Pm/+* : 1 +/⊕

With the exception of the changes induced by the irradiation of one chromosome in the X-ray series, the two series are identical. Each culture of the X-ray series contains a different irradiated chromosone, so that the series may be considered as a test of an array of typical, recoverable induced mutations. (Dominant lethals and

other drastic dominant or semidominant effects will have been eliminated or reduced in frequency; hence, the mutations available for testing will be referred to subsequently as a "random" array.) The *CyL/Pm* flies have, by virtue of the repeated backcrosses preceding the start of the experiment, a background genotype typical of the *CyL/Pm* stock present in this laboratory. The *Pm/+* flies in the two series do not differ; in neither the X-ray nor the control series is the non-Plum chromosome of these flies an irradiated chromosome.

The experimental data consist of the counts, and of the calculated ratios of *CyL/+* or *CyL/⊕*, *Pm/+*, and *+/+* or *+/⊕* flies to the *CyL/Pm* flies. These ratios were determined separately for each culture by a technique suggested by Haldane.[10] Each of the four chromosomes was studied in three experiments; in the twelve experiments the ratios in 766 control and 764 X-ray cultures were determined. The average ratios and the probabilities that the observed differences are simply the result of chance are indicated below:

Control	*CyL/+*	*Pm/+*	*+/+*
	1.094	1.146	1.008
X-ray	*CyL/⊕*	*Pm/+*	*+/⊕*
	1.115	1.137	1.033
Probability	$<.05$	$>.30$	$<.01$

The observed ratios of *Pm/+* flies, as expected, do not differ significantly. Both the *CyL/⊕* and *+/⊕* flies of the irradiated series have significantly higher ratios than their counterparts of the control series.

Distortions of Mendelian ratios in crowded cultures are generally the result of differential viabilities of the different classes of developing individuals. Indeed, this seems to be the case in the experiments reported here. Tests for factors other than differential viability which might be expected to give the observed results have proved negative. These tests have included a search for abnormal gametic ratios (*Pm/+* as compared to *Pm/⊕* males), an interaction of irradiated third or fourth chromosomes with *CyL/Pm* flies of the irradiated series (an interaction, however, which should have affected the ratio of *Pm/+* flies in this series as well), a difference in the mean culture size in the two series, a correlation between observed ratios and culture size for individual cultures, and systematic errors in classification (including those caused by nondisjunction). The details of these tests will not be given here; suffice it to say that they have yielded no alternative to the interpretation that differential viability during the larval stage has produced the observed distortions in the expected Mendelian ratios and the concomitant increase in the proportion of individuals (*CyL/⊕* and *+/⊕*) heterozygous for irradiated chromosomes.

These results are unexpected; they go beyond, for instance, the argument presented by Fisher[11] that small genetic changes should have at best, equal probabilities of being advantageous or disadvantageous. If our interpretation of these data is correct, they imply that the alleles found on typical chromosomes taken from a large interbreeding population (more precisely, our experimental *Drosophila* population No. 18) are of such a nature that, when they are made homozygous, heterozygosity for a "random" mutation is more likely to improve the viability of the otherwise homozygous individual. If one thinks in terms of proportions of loci rather than of the total probability of heterotic or nonheterotic changes at all loci, the results

suggest that a majority of loci on these chromosomes are occupied by "deleterious" alleles. The only reasonable explanation for such a preponderance of "deleterious" alleles is that they owe their existence to selection within the population on the basis of their action in heterozygous individuals.

Needless to say, these experiments are being continued. In the meantime it is important to make explicit some of the implications of these results. They indicate that one must be prepared to qualify the assertion that a "vast majority" of mutations are harmful; this statement is undoubtedly true for the effect of mutations in the homozygous condition. The results reported here do not indicate that the average mutation will improve the viability of a typical *individual* of a population. These individuals, according to the argument presented above, are already heterozygous for chromosomes containing *selected* alleles; it is difficult, then, to imagine that "random" changes will improve this nonrandom array of allelic differences. This argument applies, in fact, to highly selected, inbred lines; individuals of these lines also carry a selected constellation of genes and, although they may exhibit heterosis in crosses with other similar lines, they need not show an average heterosis when confronted with a series of random gene changes. Finally, knowing that "recessive" lethals have a slight (5 per cent ±) average deleterious effect when heterozygous, one must conclude not only that mutations with smaller viability effects have proportionately smaller average effects when heterozygous but that at some point the sign of this average effect must change; that is, the *average* heterotic effect observed must reside primarily with those mutations whose effect when homozygous is small. It remains to be seen whether this change in sign is the result of an increased probability of the occurrence of heterotic mutations among these small changes or of a more marked heterotic effect of a small number of these individual changes.

Summary.—The relative viability of two groups of flies (*D. melanogaster*) have been studied by a genetic technique. The control group was homozygous for a second chromosome (four such chromosomes were tested) obtained from a large experimental population. The second group was comparable in all respects, except that one of the pair of second chromosomes had been exposed to 500 r X-radiation; that is, this group was heterozygous for one or another of a wide variety of newly induced mutations. The results presented in this preliminary report indicate that this new, virtually unselected heterozygosity had an average heterotic effect. The implications of these results are discussed.

* This work has been done under Contract No. AT-(30-1)-557 with the United States Atomic Energy Commission.

[1] B. Wallace and J. C. King, *Am. Naturalist*, **85**, 209–222, 1951.

[2] B. Wallace and J. C. King, these PROCEEDINGS, **38**, 706–715, 1952.

[3] A. R. Cordeiro, these PROCEEDINGS, **38**, 471–478, 1952.

[4] A. R. Cordeiro and Th. Dobzhansky, *Am. Naturalist*, **88**, 75–86, 1954.

[5] Th. Dobzhansky, O. Pavlovsky, B. Spassky, and N. Spassky, *Genetics*, **40**, 781–796, 1955.

[6] C. Stern, G. Carson, M. Kinst, E. Novitski, and D. Uphoff, *Genetics*, **37**, 413–449, 1952.

[7] B. Wallace and C. Madden, *Genetics*, **38**, 456–470, 1953.

[8] N. Morton, J. F. Crow, and H. J. Muller, these PROCEEDINGS, **42**, 855–863, 1956.

[9] B. Wallace, *J. Genetics*, **54**, 280–293, 1956.

[10] J. B. S. Haldane, *J. Genetics*, **54**, 294–296, 1956.

[11] R. A. Fisher, *The Genetical Theory of Natural Selection* (Oxford: Clarendon Press, 1930), p. 38.

SPHERICAL FUNCTIONS ON A SEMISIMPLE LIE GROUP

By Harish-Chandra

DEPARTMENT OF MATHEMATICS, COLUMBIA UNIVERSITY

Communicated by Paul A. Smith, March 29, 1957

Let R be the field of real numbers and G a connected semisimple Lie group with the Lie algebra $\mathfrak{g}_0$ over R. We assume that the center of G is finite. Let K be a maximal compact subgroup of G. By a spherical function f we mean a complex-valued function on G such that $f(k_1xk_2) = f(x)$ $(k_1, k_2 \in K;\ x \in G)$. Let $\mathfrak{k}_0$ be the Lie algebra of K. Define $\mathfrak{p}_0$, $\mathfrak{h}_{\mathfrak{p}_0}$, and $\mathfrak{n}_0$ as in an earlier paper,[1] and let A and N be the analytic subgroups of G corresponding to $\mathfrak{h}_{\mathfrak{p}_0}$ and $\mathfrak{n}_0$, respectively. Then, for any $x \in G$, we denote by $H(x)$ the unique element in $\mathfrak{h}_{\mathfrak{p}_0}$ such that $x = k(\exp H(x))n$ for some $k \in K$ and $n \in N$. Introduce a linear function ρ and a polynomial function p on $\mathfrak{h}_{\mathfrak{p}_0}$ by means of the equations $e^{2\rho(H)} = \det(\mathrm{Ad}(\exp H))_{\mathfrak{n}_0}$ and $p(H) = \det(\mathrm{ad}\ H)_{\mathfrak{n}_0}$ $(H \in \mathfrak{h}_{\mathfrak{p}_0})$, where the subscript indicates the restriction on $\mathfrak{n}_0$. Then p is a product of real linear factors. Put $\pi = \alpha_1\alpha_2 \ldots \alpha_r$, where $\alpha_1, \ldots, \alpha_r$ are all the distinct prime factors of p. Let $\mathfrak{F}$ denote the space of linear functions on $\mathfrak{h}_{\mathfrak{p}_0}$. Sometimes it would be convenient to identify $\mathfrak{F}$ with $\mathfrak{h}_{\mathfrak{p}_0}$ by means of the fundamental bilinear form on $\mathfrak{g}_0$. Then π becomes a polynomial function also on $\mathfrak{F}$. Let M be the centralizer and M' the normalizer of $\mathfrak{h}_{\mathfrak{p}_0}$ in G. Then $W = M'/M$ is a finite group whose elements operate as linear transformations on $\mathfrak{h}_{\mathfrak{p}_0}$. Hence W operates also on the ring of polynomial functions on $\mathfrak{h}_{\mathfrak{p}_0}$. It can be shown that π^2 is invariant under W, and therefore $\pi^s = \varepsilon(s)\pi$ $(s \in W)$, where $\varepsilon(s) = \pm 1$. Let $\mathfrak{h}_{\mathfrak{p}_0}'$ denote the set of those points $H \in \mathfrak{h}_{\mathfrak{p}_0}$ where $\pi(H) \neq 0$. There exists a unique connected component $\mathfrak{h}_{\mathfrak{p}_0}^+$ of $\mathfrak{h}_{\mathfrak{p}_0}'$ such that $\rho(H) \geqslant \rho(sH)$ for $s \in W$ and $H \in \mathfrak{h}_{\mathfrak{p}_0}^+$. Let A_+ denote the closure of $\exp(\mathfrak{h}_{\mathfrak{p}_0}^+)$. Then $G = KA_+K$, and therefore a spherical function is completely determined by its restriction on A_+. We shall say that $H \to \infty$ $(H \in \mathfrak{h}_{\mathfrak{p}_0}^+)$ if $|\alpha_j(H)| \to \infty$ $(1 \leqq j \leqq r)$.

Put $\phi_\lambda(x) = \int_K \exp\{i\lambda(H(xk)) - \rho(H(xk))\}dk$ $(\lambda \in \mathfrak{F},\ x \in G)$, where dk is the normalized Haar measure on K. Then ϕ_λ is spherical, and it is also an elementary function of the positive-definite type.[2]

Theorem 1. *There exists a unique analytic function β on the real Euclidean space $\mathfrak{F}$ such that*

$$\lim_{H \to \infty} \left| \pi(\lambda)e^{\rho(H)}\phi_\lambda(\exp H) - \sum_{s \in W} \epsilon(s)\beta(s\lambda) \exp(i\lambda(s^{-1}H)) \right| = 0 \quad (H \in \mathfrak{h}_{\mathfrak{p}_0}^+)$$

for any $\lambda \in \mathfrak{F}$. Moreover, $|\beta(s\lambda)| = |\beta(\lambda)|$ for $s \in W$ and $\lambda \in \mathfrak{F}$.

Extend the automorphism[1] θ of $\mathfrak{g}_0$ to G, and put $n' = \theta(n^{-1})$ $(n \in N)$.

Theorem 2. *It is possible to normalize the Haar measure dn on N in such a way that*[3]

$$\beta(\lambda) = \lim_{\epsilon \to 0} \pi(\lambda_\epsilon)\int_N \exp\{-i\lambda_\epsilon(H(n')) - \rho(H(n'))\}\, dn \qquad (\epsilon > 0,\ \lambda \in \mathfrak{F}),$$

where $\lambda_\epsilon = \lambda - i\epsilon\rho$. This normalization is characterized by the condition that $\int_N e^{-2\rho(H(n'))}dn = 1$.

Define the space $\mathfrak{C}(\mathfrak{F})$ as in a previous note,[4] and for any $a \in \mathfrak{C}(\mathfrak{F})$, put $\phi_a(x) = \int_{\mathfrak{F}} \pi(\lambda)a(\lambda)\phi_\lambda(x)d\lambda$, where $d\lambda$ stands for the (suitably normalized) Euclidean measure on $\mathfrak{F}$. Then it can be shown that, for any $\mu \in \mathfrak{F}$, $\int_G |\phi_\mu(x)\phi_a(x)|\, dx < \infty$, where dx denotes the Haar measure of G. Moreover, $\beta a \in \mathfrak{C}(\mathfrak{F})$ for a in $\mathfrak{C}(\mathfrak{F})$.

THEOREM 3. *$d\lambda$ can be so normalized that*

$$\pi(\mu) \int_G (\text{conj } \phi_\mu(x))\, \phi_a(x)\, dx = |\beta(\mu)|^2 \sum_{s \in W} \epsilon(s)\, a(s\mu)$$

for $\mu \in \mathfrak{F}$ and $a \in \mathcal{C}(\mathfrak{F})$.

COROLLARY 1. $\int_G |\phi_a(x)|^2\, dx = w^{-1} \int_{\mathfrak{F}} |\beta(\lambda)|^2 \left| \sum_{s \in W} \epsilon(s) a(s\lambda) \right|^2 d\lambda$ $(a \in \mathcal{C}(\mathfrak{F}))$, *where w is the order of W.*

It is possible to show that $\int_N |\phi_a(hn)|\, dn < \infty$ for $h \in A$ and $a \in \mathcal{C}(\mathfrak{F})$.

COROLLARY 2. $e^{\rho(H)} \int_N \phi_a((\exp H)n)\, dn =$
$\int_{\mathfrak{F}} |\beta(\lambda)|^2 \{\pi(\lambda)^{-1} \sum_{s \in W} \epsilon(s)\, a(s\lambda)\}\, e^{i\lambda(H)} d\lambda$ *for $\lambda \in \mathfrak{F}$, $a \in \mathcal{C}(\mathfrak{F})$, and $H \in \mathfrak{h}_{\mathfrak{r}_0}$.*

In certain special cases it is possible to compute β explicitly. For example, β is a constant if G is complex.[6]

We shall now indicate very briefly the central idea of our method of proof. Since ϕ_λ is spherical, it can be regarded as a function on the factor space G/K. Let $\mathfrak{Q}$ be the algebra of all differential operators on G/K which are invariant under operations of G. It is known that ϕ_λ is an eigenfunction of every D in $\mathfrak{Q}$. Let $\chi_\lambda(D)$ denote the corresponding eigenvalue. Then the above results can be obtained by a detailed study of the system of differential equations $D\phi_\lambda = \chi_\lambda(D)\phi_\lambda$ $(D \in \mathfrak{Q})$.

Let $L_2(G)$ denote the Hilbert space of all square-integrable functions on G, and $I_2(G)$ the closure of the subspace consisting of those continuous spherical functions which vanish outside a compact set. Then, if it could be shown that the functions ϕ_a $(a \in \mathcal{C}(\mathfrak{F}))$ are dense in $I_2(G)$, the Plancherel formula[7] for functions on G/K would follow in an explicit form from Corollary 1 of Theorem 3.

[1] *Trans. Am. Math. Soc.*, **75**, 187–188, 1953.

[2] See *Trans. Am. Math. Soc.*, **76**, 64, 1954, Theorem 5.

[3] Here we extend π to a polynomial function on the complexification of $\mathfrak{F}$ in the obvious way.

[4] These PROCEEDINGS, **42**, 252–253, 1956.

[5] "conj c" denotes the conjugate of a complex number c.

[6] See *Trans. Am. Math. Soc.*, **76**, 253, 1954, Theorem 7.

[7] See these PROCEEDINGS, **40**, 203–204, 1954.

ON THE HOMOLOGY OF $K(\pi, n)$

BY JOHN C. MOORE*

PRINCETON UNIVERSITY

Communicated by Saunders Mac Lane, March 13, 1957

In this note we will define some little constructions in the fashion of Cartan[1] which enable the computation of the homology of the complexes $K(\pi, n)$ of Eilenberg and Mac Lane[2] for finitely generated groups π. Using these constructions, some properties of the homology and cohomology of $K(\pi, n)$ can be derived. One of the main points about these constructions is that they are constructions over the ring of p-adic integers, Z^p, and not, as usual in topology, constructions over the integers Z, or the integers modulo n, Z_n.

Let Λ be a commutative ring with unit. We will denote by $E(x, n)$ the exterior algebra over Λ with 1-generator x of dimension n, and by $\Gamma(x, n)$ the algebra

with divided powers[8] with 1-generator x of dimension n. Recall that $\Gamma(x, n)_q = 0$ for $q \not\equiv 0 \bmod n$ and that $\Gamma(x, n)_{kn}$ is a free Λ-module with basis element $\gamma_k(x)$, where $\gamma_0(x) = 1$, $\gamma_1(x) = x$, and multiplication is given by the formula $\gamma_j(x)\gamma_j(x) = (i, j)\gamma_{i-j}(x)$, where (i, j) denotes the binomial coefficient $(i + j)!/i!j!$.

Now for a fixed prime p there are four particularly interesting types of differential graded algebras which can be made by using $E(x, n)$ and $\Gamma(y, n)$. These algebras were introduced by Cartan in his study of the homology of $K(\pi, n)$. The first is the algebra $E(x, n)$ for n odd with trivial differential operator; the second is the algebra $\Gamma(x, n)$ for n even with trivial differential operator; the third is the algebra $\Gamma(x, n) \otimes E(y, n + 1)$ for n even with differential operator defined by $dx = 0$, $dy = \eta x$, where η is a power of p; and the fourth is the algebra $E(x, n) \otimes \Gamma(y, n + 1)$ for n odd with differential operator defined by $dx = 0$, $d\gamma_k(y) = \eta x\gamma_{k-1}(y)$ for $k > 0$, where η is a power of p.

We now wish to show that if $\Lambda = Z^p$, the ring of p-adic integers, and A is one of the preceding algebras, then there is an explicit algebra N such that one may define a twisted differential operator in $A \otimes N$ which makes it acyclic, induces a differential operator in N, and so that the maps f: $A \to A \otimes N$ defined by $f(a) = a \otimes 1$ and g: $A \otimes N \to N$ defined by $g(a \otimes n) = 0$ for dimension a greater than zero, and $g(1 \otimes n) = n$ are maps of differential graded algebras.

Assume now that $\Lambda = Z^p$, and let λ_i be the unit in Z^p such that $\lambda_i(p^{i-1}, p^{i-1}(p - 1)) = p$. Further, in a ring with divided powers, define $\xi_i(x) = \gamma_k(x)$, where $k = p^i - p^{i-1}$. Suppose that $A = E(x, n)$; then let $N = \Gamma(y, n + 1)$, and define $dx = 0$, $d\gamma_k(y) = x\gamma_{k-1}(y)$ for $k > 0$. The induced differential operator in N is trivial. If $A = \Gamma(x, n)$, let $N = E(a, n + 1) \otimes_{i>0} (E(a_i, p^in + 1) \otimes \Gamma(b_i, p^in + 2))$, and define $dx = 0$, $da = x$, $da_i = \gamma_{p^i}(x)$, and $db_i = pa_i - \lambda_i\xi_i(x)a_{i-1}$. The induced differential operator in N is given by $da = 0$, $da_i = 0$, and $db_i = pa_i$. Consequently, N is just a large tensor product of differential graded algebras, one of type I, the rest being of type IV. In the third case, where $A = \Gamma(x, n) \otimes E(y, n + 1)$, and $dy = \eta x$, let $N = E(a, n + 1) \otimes \Gamma(b, n + 2) \otimes_{i>0} (E(a_i, p^in + 1) \otimes \Gamma(b_i, p^in + 2))$, and define the twisted differential operator in $A \otimes N$ by $da = x$, $db = \eta a - y$, $da_i = \gamma_{p^i}(x)$, and $db_i = pa_i - \lambda_i\xi_i(x)a_{i-1}$. The induced differential operator in N is given by $da = 0$, $da_i = 0$, $db = \eta a$, $db_i = pa_i$, and again N splits into complexes of the four standard types.

Consider now the fourth case, where $A = E(x, n) \otimes \Gamma(y, n + 1)$ and $dy = \eta x$. Let $N = \Gamma(b, n + 1) \otimes E(a, n + 2) \otimes_{i>0} (E(a_i, n_i) \otimes \Gamma(b_i, n_i + 1))$, where $n_i = p^i(n + 1) + 1$. Define a differential operator in $A \otimes N$ by $db = x$, $da = \eta b - y$, $da_i = \gamma_{p^i}(\eta b - y)$, and $db_i = pa_i - \lambda_i\xi_i(\eta b - y)a_{i-1}$. The induced differential operator in N is given by $db = 0$, $da = \eta b$, $da_i = \gamma_{p^i}(\eta b)$, and $db_i = pa_i - \lambda_i\xi_i(\eta b)a_{i-1}$. This means that N does not split; however, we can make a change of basis so that it does split. Let η_i be the element in Z^p such that $\lambda_i\xi_i(\eta b) = p\eta_i\xi_i(b)$, and let $x_i = a_i - \eta_i\xi_i(b)a_{i-1}$. Then $N = \Gamma(b, n + 1) \otimes E(a, n + 2) \otimes \otimes_{i>0} (E(x_i, n_i) \otimes \Gamma(b_i, n_i + 1))$, $db = 0$, $dx_i = 0$, $da = \eta b$, $db_i = px_i$, and we have split N.

Having described these little constructions explicitly, we will now list just one theorem which can be proved by studying them.

THEOREM. Let p be an odd prime, Z the ring of integers, $Z_p = Z/pZ$, and n an odd integer. Than if $\alpha \epsilon H^{n+1}(Z_p, n; Z)$ is a generator, $\alpha^p \epsilon H^{p(n+1)}(Z_p, n; Z)$ is primitive, and is not a suspension.

* Partial support was received under Air Force Contract AF 18(600)-1494 during the period while the research summarized here was being carried out.

[1] H. Cartan, these PROCEEDINGS, **40**, 467–471, 704–707, 1954.

[2] S. Eilenberg and S. Mac Lane, "On the Groups $H(\pi, n)$. I," *Ann. Math.*, **58**, 55–106, 1953.

[3] H. Cartan, *Algèbres d'Eilenberg-Mac Lane et homotopie* (Paris, 1955).

---

*THE DIVERGENCE THEOREM WITHOUT DIFFERENTIABILITY CONDITIONS**

BY VICTOR L. SHAPIRO

RUTGERS, THE STATE UNIVERSITY, NEW BRUNSWICK, NEW JERSEY

Communicated by Deane Montgomery, March 21, 1957

The prime purpose of this note is to eliminate the differentiability conditions stated in another paper.[1] In that paper we operate in the plane, but the results are true in k-dimensional Euclidean space, $k \geqq 2$, when due consideration is paid to the boundary of the domain of the theorem.

Let D_1 be an open set in the plane. Using the notation $X = (x, y)$ and $C(X, t) =$ the circumference of the circle with radius t and center X, we define the divergence of the continuous vector field $V(X) = [A(X), B(X)]$ at the point X_0 in D_1 as the limit of $(\pi t^2)^{-1} \int_{C(X_0,t)} A\, dy - B\, dx$ as $t \to 0$, if this limit exists and is finite. Under these conditions, we call this limit div $V(X_0)$.

With this definition of divergence, the following theorem holds:

THEOREM. *Let D be a bounded domain in the (x, y)-plane, and let C be its boundary. Let E be a closed set of logarithmic capacity zero contained in $D + C$. Suppose that*

1. *The boundary C consists of one or several simple closed rectifiable curves.*
2. *The vector field $V(X) = [A(X), B(X)]$ is defined and continuous in the closure $\bar{D} = D + C$.*
3. *At each point X_0 of $D - DE$, div $V(X_0)$ exists.*
4. *Div $V(X)$ is Lebesgue integrable on D.*

Then $\int_C A(X)\, dy - B(X)\, dx = \int_D$ div $V(X)\, dX$.

To prove this theorem by a proof similar to that of the paper referred to above[1] and the uniqueness theorem (Theorem 3 of an earlier paper[2]), it is sufficient to observe that the following lemma in double trigonometric series holds:

LEMMA. *Let $A(X)$ and $B(X)$ be continuous periodic functions of period 2π in each variable with respective double Fourier series $\sum a_M e^{i(M, X)}$ and $\sum b_M e^{i(M, X)}$. Let the vector field $V(X) = [A(X), B(X)]$. Suppose that div $V(X_0)$ exists and is finite. Then*

$$\lim_{z \to 0+} \sum i(ma_M + nb_M) e^{i(M, X_0) - |M|z} = \text{div } V(X_0).$$

In the above lemma, $M = (m, n)$ represent the integral lattice points in the plane, $|M|^2 = m^2 + n^2$, and $(M, X) = mx + ny$.

In proving the above lemma, it is sufficient to take X_0 as the origin and div $V(X_0) = 0$. Then, setting

$$F(r) = \int_0^{2\pi} [A(r \cos\theta, r \sin\theta) \cos\theta + B(r \cos\theta, r \sin\theta) \sin\theta]\, d\theta, \qquad (1)$$

we have

$$F(r) = o(r) \quad \text{as} \quad r \to 0 \qquad \text{and} \qquad F(r) = 0(1) \quad \text{as} \quad r \to \infty.$$

Using well-known facts about the Poisson kernel,[3] it is easy to see that

$$\sum i(ma_M + nb_M)e^{-|M|z} = Kz \int_0^\infty \frac{r^2F(r)}{[r^2 + z^2]^{5/2}}\, dr,$$

where K is a constant.

The lemma then follows from equations (1) and the following facts:

$$z \int_0^z \frac{o(r^3)}{[r^2 + z^2]^{5/2}}\, dr = o(1) \qquad \text{as} \quad z \to 0,$$

$$z \int_\delta^\infty \frac{r^2}{[r^2 + z^2]^{5/2}}\, dr = o(1) \qquad \text{as } z \to 0,$$

$$\epsilon z \int_z^\delta \frac{r^3}{[r^2 + z^2]^{5/2}}\, dr \leqq \epsilon \qquad \text{whenever} \quad z < \delta \quad \text{and} \quad \epsilon > 0.$$

The hypotheses of the above theorem can be weakened by introducing upper and lower divergence as well as discontinuous vector fields. We shall deal with these matters in a forthcoming paper, where we shall also deal with linear negligible sets and divergence defined through squares.

* This research was supported in part by the United States Air Force through the Air Force Office of Scientific Research of the Air Research and Development Command under Contract No. AF 18(600)-1595 and in part by the Rutgers University Research Council.

[1] V. L. Shapiro, "On Green's Theorem," *J. London Math. Soc.*, **32**, 1957.

[2] V. L. Shapiro, "Generalized Laplacians," *Am. J. Math.*, **78**, 497–508, 1956.

[3] S. Bochner, "Summation of Multiple Fourier Series by Spherical Means," *Trans. Am. Math. Soc.*, **40**, 175–207, 1936.

DIFFUSION OF PARTICLES WITH MEMORY

By Peter L. Bender

PRINCETON UNIVERSITY AND NATIONAL BUREAU OF STANDARDS

Communicated by W. F. Meggers, March 1, 1957

Introduction.—In many problems in physics it is of interest to know the distribution of times that particles starting inside a given region R and subject to Brownian motion will spend inside the region before hitting the boundary. This result can be gotten directly from the solution of the diffusion equation with an absorbing boundary. In other cases the particles may retain some information concerning the length of time spent in a certain subregion $S \subset R$. It is then the distribution of times spent inside the subregion S, before hitting the boundary of R, which is of interest. One form of memory of past history might be an orientation of the magnetic moments of particles undergoing diffusion. Here the subregion S would correspond to the region in which orientation was being produced, and the absorbing

boundary would correspond to disorientation by collisions with the walls of the apparatus.

An expression for the distribution of times spent in a subregion can be given for quite general conditions. The procedure used in obtaining it is the same as that used by Kac[1] in deriving the distribution of times spent in a bounded region of Euclidean 3-space for an unrestricted Brownian motion. The result can be extended to give the distribution of times spent in R weighted according to any well-behaved function $H(x)$ of the position of the particle.

Derivation of Distribution.—Let R be a bounded open region in Euclidean N-space. The boundary Γ of R is assumed to be regular in the sense that for every point $x \epsilon \Gamma$ there is a sphere with center x, some open conical sector of which is entirely outside the closure $\bar{R}$ of R. Let S be an open subregion of R. Consider a particle starting at $x_0 \epsilon R$ at time $t = 0$ and subject to Brownian motion with Γ as an absorbing barrier; i.e., the probability $u(x, t)$ of finding the particle at x at time t satisfies the equation $\Delta u = u_t$ for homogeneous diffusion without drift and the initial and boundary conditions $u(x, 0) = \delta(x, x_0)$, $u(x, t) = 0$ if $x \epsilon \Gamma$. Further, let $K(x, y)$ be the Green's function for the differential expression $\Delta\psi$ with the boundary conditions $\psi(x) = 0$ if $x \epsilon \Gamma$.

THEOREM. *The distribution $\sigma(t)$ of times spent by the particle in S before being absorbed at Γ is given by*

$$\sigma(t) = P_r(T \leq t) = 1 - \sum_{n=1}^{\infty} c_n e^{-\lambda_n t},$$

where

$$c_n = -\lambda_n \int_S \psi_n(x)\, dx \cdot \int_S K(x_0, y)\, \psi(y)\, dy.$$

The λ_n and ψ_n are the eigenvalues and eigenfunctions of the integral equation

$$\psi(x) + \lambda \int_S K(x, y)\, \psi(y)\, dy = 0.$$

Proof: Since the region R is bounded, all moments clearly exist, and the νth moment μ_ν is given by

$$\mu_\nu = E_{x_0}\left\{\left[\int_0^\infty \chi_s[x(t)]\, dt\right]^\nu\right\}.$$

E_{x_0} means the expected value for all path functions $x(t)$ starting at x_0 at $t = 0$, and χ_s is the characteristic function of the region S. Expanding, we have

$$\mu_\nu = E_{x_0}\left\{\int_0^\infty \chi_s[x(t_1)]\, dt_1 \cdots \int_0^\infty \chi_s[x(t_\nu)]\, dt_\nu\right\}$$

$$= \int_0^\infty \cdots \int_0^\infty E_{x_0}\left\{\chi_s[x(t_1)] \cdots \chi_s[x(t_\nu)]\right\} dt_1 \cdots dt_\nu.$$

Since the $\nu!$ orderings of the t_i are equivalent, we can work with a particular one:

$$\mu_\nu = \nu! \int_{t_\nu < \infty} \int_{t_{\nu-1} < t_\nu} \cdots \int_{t_1 < t_2} E_{x_0}\left\{\chi_s[x(t_1)] \cdots \chi_s[x(t_\nu)]\right\} dt_1 \cdots dt.$$

We wish now to express the expectation value in the above expression in terms of the time-dependent Green's function $G(x, y, t)$ for the given process. This function gives the probability of going from x to y in the time t without hitting Γ. Clearly the expectation value is just the probability that $x(t_i) \in S$ for all t_i and that the particle does not hit Γ before t_3. It is given by

$$E_{x_0}\{\chi_s[x(t_1)] \ldots \chi_s[x(t_\nu)]\} = \int_S \ldots \int_S G(x_0, x_1, t_1)\, G(x_1, x_2, t_2 - t_1) \ldots G(x_{\nu-1}, x_\nu, t_\nu - t_{\nu-1})\, dx_1 \ldots dx_\nu.$$

Thus

$$\mu_\nu = \nu! \int_{t_\nu < \infty} \ldots \int_{t_1 < t_2} \int_S \ldots \int_S G(x_0, x_1, t_1)\, G(x_1, x_2, t_2 - t_1) \ldots G(x_{\nu-1}, x_\nu, t_\nu - t_{\nu-1})\, dx_1 \ldots dx_\nu\, dt_1 \ldots dt_\nu.$$

From well-known results on the spectrum of the diffusion operator (see, for example, the more general results of D. Ray[2]) we know that Mercer's theorem[3] can be used to express $G(x, y, t)$ as follows:

$$G(x, y, t) = \sum_{n=1}^{\infty} \varphi_n(x)\, \varphi_n(y) e^{-a_n t}.$$

The a_n and φ_n are the eigenvalues and eigenfunctions of the equation $\Delta\varphi + a\varphi = 0$ with the given boundary condition $\varphi(x) = 0$ if $x \in \Gamma$, and the a_n are all positive. Thus

$$\mu_\nu = \nu! \int_{t_\nu < \infty} \ldots \int_{t_1 < t_2} \int_S \ldots \int_S \sum_{(n_1, \ldots, n_\nu = 1)}^{\infty} [\varphi_{n1}(x_0)\, \varphi_{n1}(x_1) \ldots \varphi_{n_\nu}(x_{\nu-1})\, \varphi_{n_\nu}(x_\nu)] \cdot \{\exp - [a_{n_1} t_1 + a_{n_2}(t_2 - t_1) + \ldots + a_{n_\nu}(t_\nu - t_{\nu-1})]\}\, dx_1 \ldots dx_\nu\, dt_1 \ldots dt_\nu$$

$$= \nu! \sum_{(n_1, \ldots, n_\nu = 1)}^{\infty} \int_S \ldots \int_S \varphi_{n_1}(x_0)\, \varphi_{n_1}(x_1) \ldots \varphi_{n_\nu}(x_{\nu-1})\, \varphi_{n_\nu}(x_\nu)\, dx_1 \ldots dx_\nu \cdot \left\{ \int_{t_\nu < \infty} \ldots \int_{t_1 < t_2} \exp - [a_{n_1} t_1 + a_{n_2}(t_2 - t_1) + \ldots + a_{n_\nu}(t_\nu - t_{\nu-1})]\, dt_1 \ldots dt_\nu \right\}.$$

The expression in braces is equal to

$$\int_{u_1 = 0}^{\infty} \ldots \int_{u_\nu = 0}^{\infty} \exp - [a_{n_1} u_1 + a_{n_2} u_2 + \ldots + a_{n_\nu} u_\nu]\, du_1 \ldots du_\nu = (a_{n_1} a_{n_2} \ldots a_{n_\nu})^{-1}.$$

Therefore,

$$\mu_\nu = \nu! \int_S \ldots \int_S \sum_{(n_1, \ldots, n_\nu = 1)}^{\infty} \frac{1}{a_{n_1}}\, \varphi_{n_1}(x_0) \varphi_{n_1}(x_1) \ldots \frac{1}{a_{n_\nu}}\, \varphi_{n_\nu}(x_{\nu-1})\, \varphi_{n_\nu}(x_\nu)\, dx_1 \ldots dx_\nu$$

$$= \nu! \int_S \ldots \int_S \left(\sum_{n=1}^{\infty} \frac{1}{a_n}\, \varphi_n(x_0)\, \varphi_n(x_1) \right) \ldots \left(\sum_{n=1}^{\infty} \frac{1}{a_n}\, \varphi_n(x_{\nu-1})\, \varphi_n(x_\nu) \right) dx_1 \ldots dx_\nu.$$

But $\sum_{n=1}^{\infty} (-1/a_n)\ \varphi_n(x)\ \varphi_n(y) = K(x, y)$ is the Green's function for the differential operator $\Delta\psi$ with the boundary condition $\psi(x) = 0$ if $x \in \Gamma$. Thus

$$\mu_\nu = \nu! \int_S \cdots \int_S (-1)^\nu K(x_0, x_1) \cdots K(x_{\nu-1}, x_\nu)\, dx_1 \cdots dx_\nu$$

$$= \nu! \int_S \int_S (-1)^\nu K(x_0, x_1) K^{(\nu-1)}(x_1, x_\nu)\, dx_1\, dx_\nu,$$

where $K^{(\nu-1)}(x_1, x_\nu)$ is the $(\nu - 1)$st iterate of the kernel K with respect to the region S. Therefore,

$$\mu_\nu = \nu! \int_S \int_S [-K(x_0, x_1)] \sum_{n=1}^{\infty} \frac{1}{\lambda_n^{\nu-1}} \psi_n(x_1)\ \psi_n(x_\nu)\, dx_1\, dx_\nu$$

$$= \nu! \sum_{n=1}^{\infty} \left(-\frac{1}{\lambda_n^{\nu-1}}\right) \int_S \psi_n(x)\, dx \cdot \int_S K(x_0, y)\ \psi_n(y)\, dy,$$

where λ_n and ψ_n are the eigenvalues and eigenfunctions of the integral equation

$$\psi(x) + \lambda \int_S K(x, y)\ \psi(y)\, dy = 0.$$

The distribution of times spent in S can be gotten from the moments by the same method used by Kac.[1] Thus

$$\sigma(t) = 1 - \sum_{n=1}^{\infty} c_n e^{-\lambda_n t},$$

where

$$c_n = -\lambda_n \int_S \psi_n(x)\, dx \cdot \int_S K(x_0, y)\ \psi_n(y)\, dy,$$

which completes the proof.

Extensions.—If we replace $\int_0^\infty \chi_s[x(t)]\, dt$ by $\int_0^\infty H[x(t)]\, dt$ in our original formula for μ_ν, we will get the distribution, weighted according to $H(x)$, of times spent in R. The proof goes through as before for any well-behaved function $H(x)$. λ_n and ψ_n are now replaced by λ_n^* and ψ_n^*, which are the eigenvalues and eigenfunctions of the integral equation

$$\psi^*(x) + \lambda^* \int_R H(y)\ K(x, y)\ \psi^*(y)\, dy = 0.$$

The result then reads

$$\sigma^*(t) = 1 - \sum_{n=1}^{\infty} c_n^* e^{-\lambda_n^* t},$$

where

$$c_n^* = -\lambda_n^* \int_R \psi_n^*(x)\, dx \cdot \int_R H(y)\ K(x_0, y)\ \psi_n^*(y)\, dy.$$

Using Ray's results,[2] the proof also generalizes immediately to the case where particles are killed at a rate $V(x)$ throughout the region R as well as at the boundary. Only slightly more than continuity of $V(x)$ almost everywhere in R is required. Similar results are expected to hold for the elastic-barrier case.

[1] M. Kac, "On Some Connections between Probability Theory and Differential and Integral Equations," *Proc. Second Berkeley Symposium Math. Statistics and Probability*, pp. 189–215, 1951.

[2] D. Ray, "On Spectra of Second-Order Differential Operators," *Trans. Am. Math. Soc.* **77**, 299–321, 1954.

[3] R. Courant and D. Hilbert, *Methods of Mathematical Physics*, Vol. 1 (New York: Interscience Publishers, Inc., 1953).

CODES WITHOUT COMMAS

BY F. H. C. CRICK, J. S. GRIFFITH, AND L. E. ORGEL

MEDICAL RESEARCH COUNCIL UNIT, CAVENDISH LABORATORY, AND DEPARTMENT OF THEORETICAL CHEMISTRY, CAMBRIDGE, ENGLAND

Communicated by G. Gamow, February 11, 1957

This paper deals with a mathematical problem which arose in connection with protein synthesis. We present the solution here because it gives the "magic number" 20, so that our answer may perhaps be of biological significance. To make this clear, we sketch in the biochemical background first.

It is assumed in one of the more popular theories of protein synthesis that amino acids are ordered on a nucleic acid strand (see, for example, Dounce[1]) and that the order of the amino acids is determined by the order of the nucleotides of the nucleic acid. There are some twenty naturally occurring amino acids commonly found in proteins, but (usually) only four different nucleotides. The problem of how a sequence of four things (nucleotides) can determine a sequence of twenty things (amino acids) is known as the "coding" problem.

This problem is a formal one. In essence, it is not concerned with either the chemical steps or the details of the stereochemistry. It is not even essential to specify whether RNA or DNA is the nucleic acid being considered. Naturally, all these points are of the greatest interest, but they are only indirectly involved in the formal problem of coding.

The first definite proposal was made by Gamow.[2] His code, which was suggested by the structure of DNA, was of the "overlapping" type. The meaning of this is illustrated in Figure 1. Gamow's code was also "degenerate"—that is, several sets of three letters (picked in a special way) stood for a particular amino acid. However, all the 64 ($4 \times 4 \times 4$) possible sets of three letters stood for one amino acid or another, so that any sequence whatever of the four letters stood for a definite sequence of amino acids.

It is easy to see that codes of the overlapping type impose severe restrictions on the allowed amino acid sequences. Unfortunately, no such restrictions have been found, although considerable (unpublished) efforts have been made, by a number of workers, to find them. Part of this work has been reviewed by Gamow, Rich,

and Yčas.[3] However, the amino acid sequences so far determined experimentally are of limited extent, and it is possible that there may be restrictions on the neighbors of the rarer amino acids, such as tryptophan. Thus, while overlapping codes seem highly unlikely, partial overlapping is not impossible. At the moment, however, nonoverlapping codes seem the most probable, and these are the only ones we shall consider here.

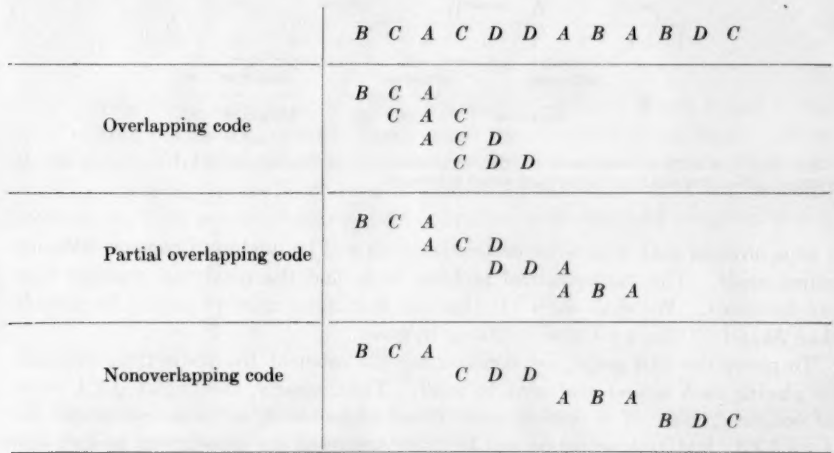


FIG. 1.—The letters *A*, *B*, *C*, and *D* stand for the four bases of the four common nucleotides. The top row of letters represents an imaginary sequence of them. In the codes illustrated here each set of three letters represents an amino acid. The diagram shows how the first four amino acids of a sequence are coded in the three classes of codes.

If each amino acid were coded by *two* bases (rather than the three shown in Fig. 1), we should only be able to code $4 \times 4 = 16$ amino acids. It is natural, therefore, to consider nonoverlapping codes in which *three* bases code each amino acid. This confronts us with two difficulties: (1) Since there are $4 \times 4 \times 4 = 64$ different triplets of four nucleotides, why are there not 64 kinds of amino acids? (2) In reading the code, how does one know how to choose the groups of three? This difficulty is illustrated in Figure 2. The second difficulty could be overcome by reading off from one end of the string of letters, but for reasons we shall explain later we consider an alternative method here.

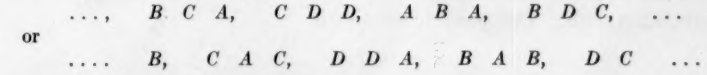


FIG. 2.—The commas divide the string of letters into groups of three, each representing one amino acid. If the ends of the string of letters are not available, this can be done in more than one way, as illustrated. The problem is how to read the code if the commas are rubbed out, i.e., a comma-less code.

We shall assume that there are certain sequences of three nucleotides with which an amino acid can be associated and certain others for which this is not possible. Using the metaphors of coding, we say that some of the 64 triplets make sense and

some make nonsense. We further assume that all possible sequences of the *amino acids* may occur (that is, can be coded) and that at every point in the string of letters one can only read "sense" in the correct way. This is illustrated in Figure 3. In other words, any two triplets which make sense can be put side by side, and yet the overlapping triplets so formed must always be nonsense.

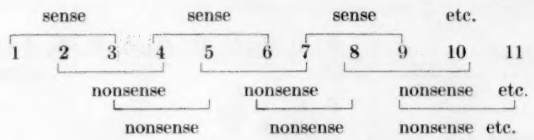


FIG. 3.—The numbers represent the positions occupied by the four letters *A*, *B*, *C*, and *D*. It is shown which triplets make sense and which nonsense.

It is obvious that with these restrictions one will be unable to code 64 different amino acids. The mathematical problem is to find the maximum number that can be coded. We shall show (1) that the maximum number cannot be greater than 20 and (2) that a solution for 20 can be given.

To prove the first point, we consider for the moment the restrictions imposed by placing each amino acid next to itself. Then, clearly, the triplet *AAA* must be nonsense, since, if it corresponded to an amino acid, α, then $\alpha\alpha$ would be *AAAAAA*, and this sequence can be misinterpreted by associating α with the second to fourth, or third to fifth, letters. We can thus reject *AAA*, *BBB*, *CCC*, and *DDD*.

It is easy to see that the 60 remaining triplets can be grouped into 20 sets of three, each set of three being cyclic permutations of one another. Consider as an example *ABC* and its cyclic permutations *BCA* and *CAB*. It is clear that we can choose any one of these, but not more than one. For suppose that we let *BCA* stand for the amino acid β; then $\beta\beta$ is *BCABCA*, and so *CAB* and *ABC* must, by our rules, be nonsense. Since we can choose at the most one triplet from each cyclic set, we cannot choose more than 20. No solution is possible, therefore, which codes more than 20 different amino acids.

We have so far not considered the effects of putting unlike amino acids together, to give pairs of the form $\alpha\beta$ and $\beta\alpha$. It might be thought that this would still further reduce the possible number of amino acids, but this turns out not to be so, since we can write down a construction which obeys all our rules and yet codes 20 different amino acids. One possible solution is

$$A\ B\ \begin{matrix}A\\B\end{matrix} \qquad \begin{matrix}A\\B\end{matrix}\ C\ \begin{matrix}A\\B\\C\end{matrix} \qquad \begin{matrix}A\\B\\C\end{matrix}\ D\ \begin{matrix}A\\B\\C\\D\end{matrix}$$

where $A\ B\ \begin{matrix}A\\B\end{matrix}$ means *ABA* and *ABB*, etc. It is easy to see, by systematic enumer-

ation, that one can place any two triplets of this set next to each other without producing overlapping triplets which belong to the set.

The solution given above is not unique. Another satisfactory choice of 20 allowed sequences is

$$\begin{array}{cccccccccc} B & A & A & & & & & & & \\ & & & A & & A & & A & & A \\ & & & B & C & B & D & B & & C \\ & & & & & C & & C & & D \\ A & B & B & & & & & & & \end{array}$$

If we exclude trivial variations, such as permuting letters (e.g., A into C and C into A) or writing the code backwards, there are at least 8 different solutions. These can be obtained by taking one or the other of the two solutions given above and reversing either the entire second set of triplets, or the entire third set, or both. For example, if we reverse the second set of triplets in the first solution given above, we obtain the solution

$$\begin{array}{cccccccccc} & & & A & & & & A & & A \\ A & B & A & B & C & A & & B & D & B \\ & & B & C & & B & & C & & C \\ & & & & & & & & & D \end{array}$$

If we enumerate *all* solutions we have been able to find, including the variations produced by interchanging letters and reversing the direction of the code, we obtain a total of 288 solutions (192 from variants of the first solution above and 96 from variants of the second one).

The problem we have considered is a special case of the more general situation in which one Greek letter is determined by n Roman letters selected from a total of m different Roman letters. One can obtain an upper limit for the number of possible Greek letters by the methods we have used, but it is not in general easy to see whether this upper limit can be achieved. One can easily see by trial that the upper limit of six, corresponding to $n = 2$, $m = 4$, cannot be achieved, only five Greek letters being possible; hence the upper limit cannot be achieved for $n = 2$, $m > 4$, either. The solution for $n = 3$ and arbitrary m is

$$\begin{array}{cccccccccc} & & & & & & & & & A \\ & & & & & & & A & & B \\ & & & & & & & B & & C \\ A & B & A & & A & & A & C & K & . \\ & & B & & B & C & B & . & & . \\ & & & & & & C & . & & . \\ & & & & & & & L & & L \\ & & & & & & & & & M \end{array}$$

or, more concisely, writing $A_1 A_2 \ldots A_m$ for the nucleotides, then a solution which attains the upper limit is the set of triplets $A_i A_j A_k$ for all $i, j, k = 1, 2, \ldots, m$, satisfying $k \leqslant j, i < j$. We have not solved the general problem.

A Physical Interpretation.—To fix ideas, we shall describe a simple model to illustrate the advantages of such a code. Imagine that a single chain of RNA, held in

a regular configuration, is the template. Let the intermediates in protein synthesis be 20 distinct molecules, each consisting of a trinucleotide chemically attached to one amino acid. The bases of each trinucleotide are chosen according to the code given above. Let these intermediate molecules combine, by hydrogen bonding between bases, with the RNA template and there await polymerization. Now imagine that such an amino acid–trinucleotide were to diffuse into an incorrect place on the template, such that *two* of its bases were hydrogen-bonded, though not the third. We postulate that this incomplete attachment will only retain the intermediate for a very brief time (for example, less than 1 millisecond) before the latter breaks loose and diffuses elsewhere. However, when it eventually diffuses to the correct place, it will be held by hydrogen bonds to all three bases and will thus be retained, on the average, for a much longer time (say, seconds or minutes). Now the code we have described insures that this more lengthy attachment can occur only at the points where the intermediate is needed. If one of the 20 intermediates could stay for a long time on one of the false positions, it would effectively block the two positions it was straddling and hold up the polymerization process. Our code makes this impossible. This scheme, therefore, allows the intermediates to accumulate at the *correct* positions on the template without ever blocking the process by settling, except momentarily, in the wrong place. It is this feature which gives it an advantage over schemes in which the intermediates are compelled to combine with the template one after the other in the correct order.

The example given here is only for illustration, but it brings out the physical idea behind the concept of a comma-less code.

In passing, it should be mentioned that while the idea of making three nonoverlapping nucleotides code for one amino acid at first sight entails certain stereochemical difficulties, these are not insuperable if it is assumed that the polypeptide chain, *when polymerized*, does not remain attached to the template. A detailed scheme along these lines has been described to us by Dr. S. Brenner (personal communication).

General Remarks.—The arguments and assumptions which we have had to employ to deduce this code are too precarious for us to feel much confidence in it on purely theoretical grounds. We put it forward because it gives the magic number—20—in a neat manner and from reasonable physical postulates. It should be noted, however, that other codes can be derived which restrict the amino acids to 20, in particular the "combination code" of Gamow and Yčas,[4] though we regard the physical assumption underlying their code as implausible. Some direct experimental support is therefore required before our idea can be regarded as anything more than a tentative hypothesis.

Summary.—The problem of how, in protein synthesis, a sequence of four things (nucleotides) determines a sequence of many more things (amino acids) is known as the coding problem. We consider codes involving nonoverlapping triplets of nucleotides, each triplet coding for one amino acid. We show that to allow all possible amino acid sequences without giving false readings of the code (due to reading the last part of one triplet and the first part of the next), we must limit the number of kinds of amino acids which the code can handle. We prove that an upper bound is 20 and show that a code for 20 can in fact be written down. It is

well known that 20 is the number found experimentally. The physical ideas behind such a code are briefly discussed.

[1] A. L. Dounce, *Enzymologia*, **15**, 251, 1952.
[2] G. Gamow, *Nature*, **173**, 318, 1954; *Kgl. Danske Videnskab. Selskab Biol. Medd.*, **22**, 3, 1954.
[3] G. Gamow, A. Rich, and M. Yčas, *Advances in Biol. and Med. Physics*, Vol. 4 (New York: Academic Press Inc., 1955).
[4] G. Gamow and M. Yčas, these PROCEEDINGS, **41**, 1011, 1955.

STRAIN ELECTROMETRY AND CORROSION I. GENERAL CONSIDERATIONS ON INTERFACIAL ELECTRICAL TRANSIENTS

BY ALBERT G. FUNK, J. CALVIN GIDDINGS, CARL J. CHRISTENSEN, AND HENRY EYRING

CHEMISTRY DEPARTMENT, UNIVERSITY OF UTAH, SALT LAKE CITY, UTAH

Communicated by H. Eyring, March 18, 1957

Introduction.—Chemical and physical processes occurring in the neighborhood of metal-solution interfaces determine the corrosive properties of metals. Certain of those processes that involve charge transfer are responsible for observed electrode potentials. Conversely, the electrode potential is an accurate and sensitive measure of the processes that give it birth.

Electrode-potential measurements yield information on corrosive properties under a variety of circumstances. The dissolution of metals can be studied as it depends upon salt concentrations, pH, presence of gases, temperature, and metal impurities. An important class of phenomena is observed when, in addition to the other factors, the electrode is plastically deformed. The deformation changes the electrode properties, and reactions proceed rapidly (approximately 1 second) in such a direction as to restore the original potential. The measurement of the resulting electrical transients, strain electrometry, has been used to piece together a kinetic picture of the underlying corrosion process. A great deal of experimental and theoretical work must still be done to isolate and identify all the important kinetic steps and to show how their rates change with concentrations, pH, etc. However, some general features of these corrosion transients are clear, and these are presented along with some of the pertinent experimental data. A detailed account of this work will appear elsewhere.

A number of workers have measured electrode strain transients under a variety of conditions. Dudley, Elliot, McFadden, and Shemilt[1] and Gautam and Jha[2] measured the electrode strain transients of copper wire in aqueous solutions. Similar measurements have been reported by Nikitin[3] and Zaretskii[4] on copper, silver, iron, magnesium, and aluminum. Coffin and Simon[5] found the voltage transients of a creeping zinc crystal, while Fryxell and Nachtrieb[6] experimented with metallic gold and silver. Some of the above results are contradictory; others serve as a valuable guide for the respective topics covered. Since our measurements on copper are more extensive than any of the above, these references are acknowledged but not widely quoted.

Measurements of electrode strain transients of copper have been made, along with exploratory measurements involving nickel, iron, aluminum, silver, manganum, and nichrome. Two electrodes, both of the same substance, are immersed in the solution of a single cell. The amount of strain is controlled by adding designated weights to one of the electrodes. The potential, then, is always measured so that only the transients are observed, and these decay to a value near zero in a matter of seconds. It is to be noted that these potentials are not referred to those of a standard cell. This work—the electrode potentials of corroding metals—has itself been the subject of extensive research.[7]

The potential-time curves of strain electrometry depend strongly upon ion concentrations in solution. Some examples are shown in Figure 1. For most substances the curves are similar to the one shown for a neutral solution. These

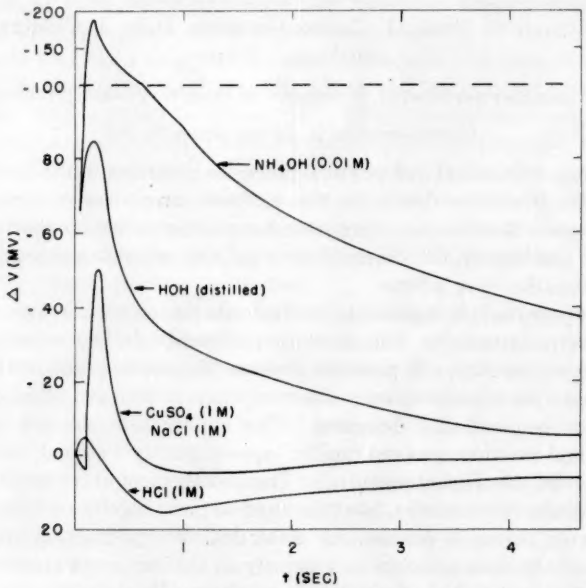


FIG. 1.—Examples of electrode strain transients.

curves may conveniently be described in terms of three parameters: the maximum voltage, the elapsed time between the application of strain and the maximum voltage (growth time), and the time in which the potential decays from its maximum value to half of this (decay time).

The behavior in the case of strong acids, with two peaks, is unusual but not unique. Over a wide concentration range, NaCN solutions behave similarly, with an anodic (negative) potential maximum preceding a cathodic (positive) maximum. The situation is reversed in the case of a solution composed of a mixture of NaCl and $CuSO_4$, each at unit molarity. In this case a small cathodic peak precedes a larger anodic maximum. However, a great majority of solutions yield but a single anodic peak, although there is a large variation in the parameters that describe these transients. Thus values for the maximum voltage range from a few milli-

volts (1M $CuSO_4$) to nearly 200 millivolts (0.01M NH_4OH), and the decay times range from 0.15 second (1M $CuCl_2$) to minutes in strong bases.

Theory.—At the steady-state potential of a given electrode, anodic and cathodic processes transfer charge across the electrical double layer at the same rate. Further, each anodic reaction has as its reverse a cathodic reaction, and the steady-state potential, obtained when the two rates are equal, may be termed the "pair potential" for the given reaction. In the case of corroding metals in aerated aqueous solution two reaction pairs have been found adequate to explain most experimental electrode data. The potentials of these pairs, the cathodic oxygen electrode (*1*) and anodic metal-metal ion (*2*) pairs, are shown schematically in Figure 2, along with other steady-state potentials.

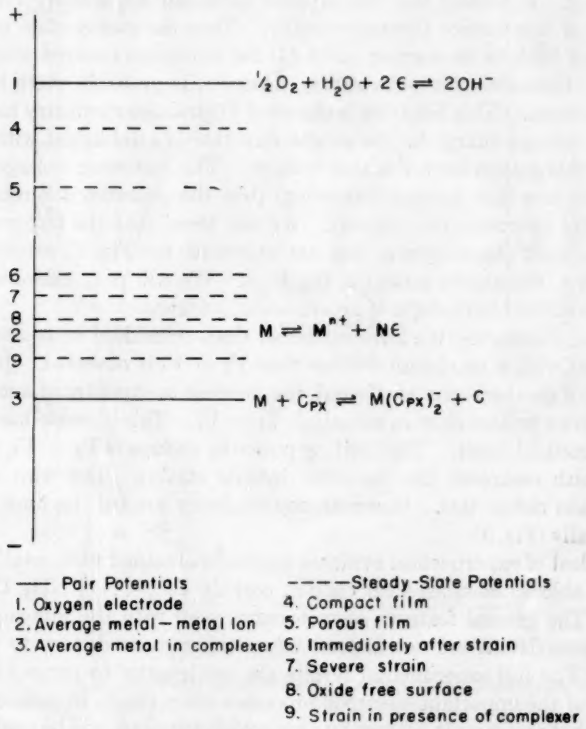


FIG. 2.—Schematic illustration of pair potentials and steady-state potentials.

In cases where only two reaction pairs are important, the steady-state potential lies between the pair potentials, and closer to that pair potential established by the faster reactions. Under usual conditions, the oxygen electrode reaction is slow (due to a large overvoltage effect), as is the metal-metal ion reaction (due to the slow diffusion through the oxide layer of the metal ion). The steady-state potential, then, may not be near either of the pair potentials.

The steady-state potential is strongly influenced by the nature of the oxide film,

since, as mentioned, diffusion of metal ions through this film controls the reaction of a metal to form its ions. If the film is compact, the diffusion is very slow, and the potential is established at *4* (Fig. 2), nearer the oxygen electrode potential (*1*). A porous film does not offer such resistance to diffusion, and the steady-state potential in this case is the more anodic value shown as *5*. If an oxide film is not present, the metal-metal ion reaction proceeds rapidly, establishing a potential (*8*) near the pair potential (*2*).

When an electrode is plastically strained, the brittle oxide film is presumably ruptured, exposing bare, or near-bare, metal surface.[8] This is tantamount to the destruction of the diffusion barriers in these ruptured regions, so that the anodic reaction pair (*2*) equilibrates rapidly. The new steady-state potential is shown as *6* in Figure 2. It is clear that the exposed metal surface is short-lived, since the reoxidation of this surface proceeds rapidly. Thus the steady-state potential will decay from *6* back to its starting point (*4*) for electrodes covered with a compact oxide film. Concentration polarization, however, is probably more important in the decay process. This behavior is observed in strain-electrometry measurements (Fig. 1); a voltage charge in the anodic direction is established with the tensile stress, and this decays toward a zero voltage. The maximum voltage is expected to be slightly less (due to capacitance lag) than the potential difference, $V_6 - V_4$ (compact-film electrode, i.e., copper). We see, then, that the film-rupture model explains the most characteristic (but not universal; see Fig. 1) property of strain electrometry: the anodic potential transient. We will next examine this model in greater detail and in the light of experimental evidence.

Supporting Evidence.—We have suggested that, coincident with strain, a potential transient with a maximum voltage near $V_6 - V_4$ is observed. If, in another experiment, a greater strain is effected, the increase in exposure of anodic area will lead to an even greater drop in potential, $V_7 - V_4$. This increase has a logical, as well as a practical, limit. The limiting potential change is $V_8 - V_4$, a value that coincides with complete film removal (infinite strain). The wire electrode, of course, breaks before this. However, the tendency toward the limit can be seen experimentally (Fig. 3).

A great deal of experimental evidence has been obtained with solutions containing agents able to complex with copper, notably Cl^-, CN^-, NH_3, $C_4H_4O_6^-$, and pyridine. The general features seem to agree well with the film-rupture model, although some details are not understood; nothing contradictory to the model is apparent. The full experimental results are too lengthy to present here. However, some of the important electrode processes are common to most of these complexers, so that an example, ammonium hydroxide solutions, will be used to illustrate the general pattern.

Visual inspection shows us that a concentrated NH_4OH solution will quickly remove an oxide film from the surface of copper. As succeeding solutions are made more dilute, the film is removed more slowly, then finally not at all. In the experiments the wire electrode is allowed to stand in solution approximately 2 minutes before the stress is applied. It is found that solution of concentration greater than about 10^{-2} molar NH_4OH will remove a major portion of the oxide film in 2 minutes. Experimental data shows that a maximum in the voltage-concentration curve appears near 10^{-2} molar NH_4OH (see Fig. 4). In more dilute solutions the

potential falls to the value for water, and to the right (more concentrated) side of the maximum the potential plunges to values near zero.

Again Figure 2 can be used to explain these results. With concentration increase, the potential of the anodic pair is lowered to *3*, due to depletion of metal ions by the NH_3 molecules. When strain ruptures the film, the steady-state potential falls to V_9, a value characteristic of the extremely anodic, exposed area. The potential transient in this case has a maximum value near $V_9 - V_4$, larger than the value $V_6 - V_4$ at zero concentration. For this reason, the potential increases with concentration until 10^{-2} molar, where the film itself begins to dissolve. When the

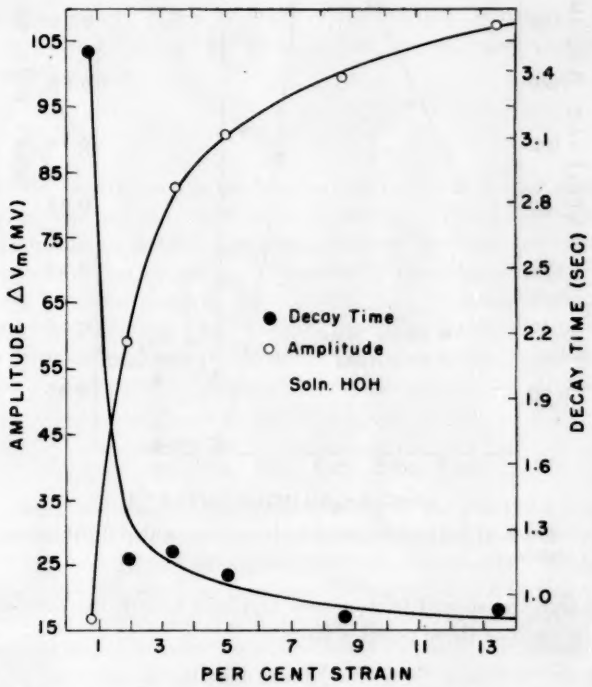


FIG. 3.—Amplitude and decay time as functions of per cent strain.

film is partially dissolved to begin with, the potential no longer starts at the value V_4 but at a much more negative value. The potential difference decreases rapidly beyond this point, as the film is more completely peptized, and the starting potential approaches V_9.

General Theory.—Competing anodic and cathodic reactions fix the potential of an electrode, as indicated in the discussion of Figure 2. In this section we will present a theoretical treatment of these processes. For purposes of generality, all possible charge-transferring reactions must be considered, although in practice many of these may be neglected. Each anodic reaction has a velocity (number of reactions per second per unit area), v_i, and the charge transfer (per second per unit

area) from the electrode to the solution, due to this reaction, is $z_i e v_i$. The reverse of the anodic reaction is a cathodic reaction, proceeding with the velocity, v_i'.

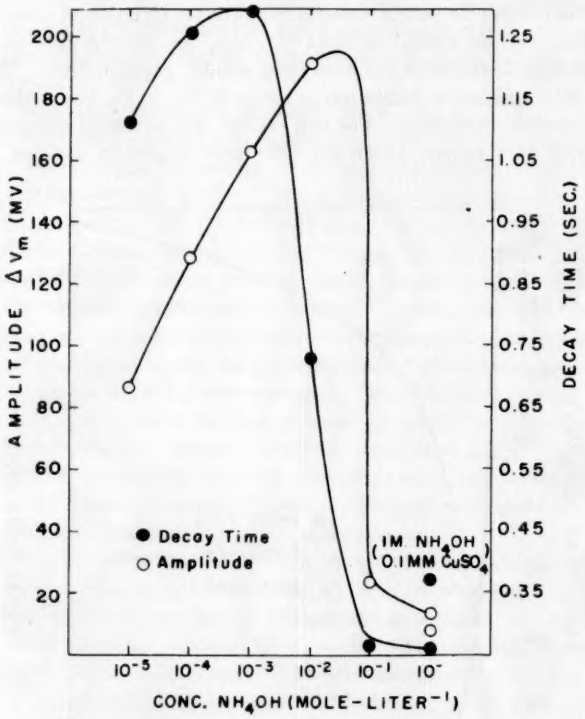


FIG. 4.—Change of amplitude and decay time with NH_4OH concentration.

The total charge transferred in unit area and unit time is the sum of the charge transferred by each of these possible pairs,

$$Q = e \sum_i z_i (v_i - v_i'). \tag{1}$$

The electron charge is represented as e, and the number of electron charges transported by a single ith process, irrespective of sign, by z_i. The reaction velocities are voltage dependent, and a relationship of the following form can often be written:

$$v_i = v_{i_0} \exp\left(\frac{z_i \alpha_i e V}{kT}\right) \quad v_i' = v_{i_0}' \exp\left(\frac{-z_i(1 - \alpha_i)eV}{kT}\right), \tag{2}$$

where V is the potential difference between electrode and solution, and α_i is a fraction, which in simple cases represents the change in free energy, due to potential, between the initial and activated state, divided by the change between initial and final states. More will be said about this term later.

It is instructive to write the charge flux in equation (1) as $d(CV)/dt$, where C is

the capacitance of unit area. Hence equation (1) may be written as a differential equation describing the change of potential, V, with time.

$$\frac{d(CV)}{dt} = e\sum z_i \left[v_{i_0} \exp\left(\frac{z_i \alpha_i e V}{kT}\right) - v_{i_0}' \exp\left(\frac{-z_i(1-\alpha_i)eV}{kT}\right)\right]. \quad (3)$$

This equation for the electrical transient, with the appropriate reaction velocities inserted, serves to describe the potential dependence of the various cases shown in Figure 1.

Initially (before the electrode is strained), the potential is stationary, so that the left member of equation (3) equals zero. Thus equation (3) becomes an algebraic equation in which V is a function of the reaction velocities. In the particular case in which all the z_i's are equal, and all the α_i's equal $1/2$, the stationary solution of equation (3) is

$$V(\text{initial}) = \frac{kT}{ze} \ln \frac{\sum v_{i_0}'}{\sum v_{i_0}}. \quad (4)$$

The point of the maximum change from initial potential is also stationary with respect to potential and can be treated as above. The difference between the initial and the maximum potential is a result of the altered reaction velocities. For convenience, the reaction velocities at the point of maximum change will be identified as u_i and u_i', whereas v_i and v_i' will be retained for the initial reaction velocities. Thus an expression, identical with equation (4) except for the altered reaction velocities, can be obtained for $V(\text{maximum})$. The maximum potential change, or the amplitude of the transient, $V(\text{maximum}) - V(\text{initial}) = \Delta V_m$, is found to be

$$\Delta V_m = \frac{kT}{ze} \ln \frac{\sum u_{i_0}' \sum v_{i_0}}{\sum v_{i_0}' \sum u_{i_0}}. \quad (5)$$

We have mentioned specific examples of some of the reactions responsible for strain transients: they are sometimes diffusion-controlled, and in other cases passage over a single energy barrier controls the reaction velocity. A theory that is applicable to all such processes has been outlined by Parlin and Eyring.[9] By means of this, the voltage dependence of any reaction velocity can be obtained, provided that the potential energy is known as a function of distance. The reader is referred to the original work for the general equation for such reactions; we will concern ourself here with certain special cases of interest.

In order that charge transfer can be effected, charged particles must cross a series of potential energy barriers. It is often the case that a single energy barrier is much higher than any other. In this case the general theory reduces to a result obtained previously.[10] If the electrical potential difference between the end and the beginning of these barriers is V, then αV is the potential drop between the single highest barrier and the initial equilibrium position. Equation (2) describes this model without modification.

The velocity of a reaction is often determined by a series of potential energy barriers, all of equal height. Diffusion processes (as in oxide films) can be accounted for in this manner. For this example, the reaction velocity can be written (cf. Parlin and Eyring,[9] eq. [12]) as

$$v_i = \frac{\lambda k_{i_0} c_i g}{n} \exp\left(\frac{-z_i eV}{2kT}\right),$$

$$\frac{g}{n} = \frac{\exp\ (-z_i eV/2nkT) - \exp\ (z_i eV/2nkT)}{\exp\ (-z_i eV/2kT) - \exp\ (z_i eV/2kT)}, \tag{6}$$

where the n barriers have been assumed an equal distance, λ, from one to another. The term k_{i_0} is the value of the rate constant for passage over a single one of the barriers when the potential, V, is zero, and c_i is the concentration of the ith species just in front of the energy barriers. The electrical energy expended in crossing a single barrier, $z_i eV/n$, is nearly always small compared with kT. This makes permissible the expansion of the exponentials in the numerator of g. Two particular cases arise, depending upon the potential, V. For small values of V ($z_i eV \ll kT$), the exponential in the denominator of g/n can also be expanded. This leads to a reaction velocity

$$v_i = \frac{\lambda k_{i_0} c_i}{n} \exp\left(\frac{-z_i eV}{2kT}\right), \tag{7}$$

where, of course, the remaining exponential may also be expanded. This is of the same form as equation (2), with $v_{i_0} = \lambda k_{i_0} c_i/n$, and $\alpha = 1/2$.

With larger potentials ($z_i eV \gg kT$, $z_i eV \ll nkT$), a potential dependence different from equation (2) is found. When the potential difference between solution and electrode is positive, the anodic reaction velocity can be approximated by

$$v_i = v_{i_0} \frac{z_i eV}{kT} \exp\left(\frac{-z_i eV}{kT}\right), \tag{8}$$

and the cathodic reaction velocity becomes

$$v_i = v_{i_0} \frac{z_i eV}{kT}. \tag{9}$$

This differs from the form of equation (2), since V appears in the pre-exponential term. However, since a large V was stipulated, the exponential term is far more important. Hence this resembles equation (2), with $\alpha = 1$. (For values of the potential which are negative, α becomes equal to 0. It is to be remembered that z_i is defined to be positive, irrespective of the sign of the ion.)

The above special forms of the reaction velocity and its potential dependence are valuable in assessing the accuracy of equation (5), which in turn, was derived with the use of equation (2), $\alpha = 1/2$. We conclude that equation (5) may often be considered a good approximation (particularly with small values of V) and that in other cases it may be used as a simple first approximation. Future work will involve the more specific application of equation (5).

The authors would like to acknowledge a grant from the Office of Naval Research, Contract No. N7 onr 45103, in support of this work.

[1] R. S. Dudley, R. Elliott, W. H. McFadden, and L. W. Shemilt, *J. Chem. Phys.*, **23**, 585, 1955.

[2] L. R. Gautam and J. B. Jha, *Proc. Indian Acad. Sci.*, *A*18, 350, 1953.
[3] L. V. Nikitin, *J. Gen. Chem.* (*U.S.S.R.*), 11, 146, 1941.
[4] E. M. Zaretskii, *Zhur. Priklad. Khim.*, 24, 614, 1951.
[5] F. D. Coffin and S. L. Simon, *J. Appl. Phys.*, 24, 1333, 1953.
[6] R. E. Fryxell and N. H. Nachtrieb, *J. Electrochem. Soc.*, 99, 495, 1952.
[7] O. Gatty and E. C. R. Spooner, *The Electrode Potential Behaviour of Corroding Metals in Aqueous Solutions* (Oxford: Clarendon Press, 1936).
[8] Oxide-film rupture may be accomplished by application of tensile stress or torsional stress or by scratching the surface with a glass rod. These disturbances all lead to a potential change in the same direction and of similar magnitude and time dependence.
[9] R. B. Parlin and H. Eyring, *Ion Transport across Membranes* (New York: Academic Press, Inc., 1954), p. 103.
[10] S. Glasstone, K. J. Laidler, and H. Eyring, *The Theory of Rate Processes* (New York: McGraw-Hill Book Co., Inc., 1941), chap. 10.

VARIATION OF REACTION RATE WITH VIBRATIONAL STATE

BY WILLIAM KLEMPERER AND DUDLEY HERSCHBACH*

DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY, CAMBRIDGE, MASSACHUSETTS

Communicated by G. B. Kistiakowsky, February 8, 1957

Chemical reactions in general are studied with systems whose components have a thermal distribution. The interpretation of such experiments requires averaging elementary reactions over many initial quantum states. Very little can be inferred about the relative reactivity of molecules in the individual quantum states beyond the rough indication provided by the variation of the reaction rate with temperature. A much more direct and fundamental type of information comes from experiments in which at least one of the participating species is sorted into known states of quantization before reaction.

A method presently available for the selection of states is high-resolution molecular-beam spectroscopy. This note is concerned with the reaction commonly used for the detection of alkali halide beams—dissociation and ionization on a hot tungsten surface. Evidence will be presented which indicates that the rate of dissociation depends on the vibrational state of the incident molecules.

The Beam Experiment.—Marple and Trishka[1] have recently studied the radio-frequency spectrum of lithium chloride by the molecular-beam electric-resonance method. In their apparatus (see Fig. 1), molecules in the state of angular momentum $J = 1$ and space quantization $M_J = 1$ are selected by an inhomogeneous de-

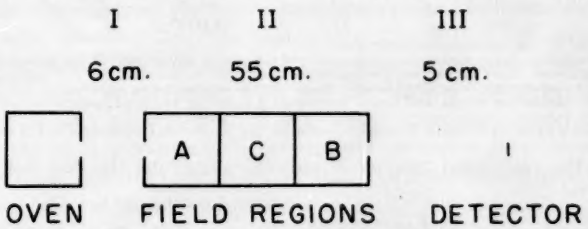


FIG. 1.—Schematic diagram of the apparatus of Marple and Trischka.

flecting field A. In the region of the homogeneous electric field C, transitions to the state $J = 1$, $M_J = 0$ are induced by monochromatic radio-frequency radiation. A second inhomogeneous field B then deflects only the molecules with $J = 1$, $M_J = 0$ to the tungsten-wire detector. The frequency for the electric hyperfine transition is slightly dependent upon the vibrational state of the molecule, and the high resolution permits separate resonances to be observed for four different vibrational states, $v = 0, 1, 2, 3$. Thus, except for background from scattered molecules and for molecules which change their state by radiation processes between leaving the last inhomogeneous field B and reaching the detector, the molecules reaching the tungsten wire for a given resonant frequency of the r.f. field are in a completely specified state with respect to electronic energy (ground state), vibrational energy (v, as selected by the r.f.), angular momentum ($J = 1$), and space orientation of the angular momentum ($M_J = 0$).

Reaction of LiCl on a Tungsten Wire.—The over-all reaction taking place at the hot tungsten wire is

$$LiCl = Li^+ + Cl + e^-. \qquad (1)$$

The current of lithium ions is detected with a mass spectrometer and ion multiplier. In this "flip-in" type of beam experiment only about 10^3 molecules per second reach the detector,[2] so that the number of those which interact with each other is negligible. The tungsten wire is estimated[2] to be at 1,400 ± 200° K. (At this temperature the surface is actually covered with a monolayer of molecular oxygen.[3])

Table 1 gives the relative populations of vibrational states calculated from the oven temperature and the known vibrational frequency,[4] under the assumption that the molecules leaving the oven are harmonic oscillators in thermal equilibrium. Spontaneous vibrational transitions during transit slightly alter the relative numbers reaching the detector, as shown.

The relative ion currents observed by Marple and Trischka for the hyperfine resonances corresponding to molecules in different vibrational states are listed in the fourth column of Table 1. It is seen that the relative intensities I_v are not proportional to the calculated number of molecules reaching the detector. The data is well fitted by

$$\frac{I_v}{N_v} = (1.4)^v = \exp(0.34v). \qquad (2)$$

TABLE 1
COMPARISON OF VIBRATIONAL STATES

VIB. QUANT. NUMBER v	REL. POP. LEAVING OVEN* N_v'	REL. POP. REACHING WIRE N_v	OBS. REL. ION CURRENT I_v	I_v/N_v Obs.	I_v/N_v Calc.†
0	1	1	1	1	1
1	0.28 ± 0.04	0.27	0.38	1.4	1.4
2	0.078 ± 0.02	0.075	0.15	2.0	2.1 ± 0.07
3	0.022 ± 0.01	0.020	0.058	2.9	3.3 ± 0.2
Effective vib. temp.‡	805	772	1,074° K.		

* For 805 ± 100° K.; the uncertainties apply also to N_v.

† Only the uncertainty due to that in the temperature of the wire (1,400 ± 200° K.) is indicated. It should be noted that if a different value for the oven temperature were used to calculate N_v, the experimental values of I_v/N_v would differ considerably from those listed. However, the corresponding adjustment of k_v/k to fit I_1/N_1 of course changes the theoretical values, and similar agreement is found for any oven temperature in the range 700°–900° K.

‡ The temperature found by fitting a Boltzmann factor for the vibrational frequency $\nu = 715$ cm^{-1}.

If (merely for characterization) a Boltzmann factor is fitted to the relative ion currents $I_v = \exp(-hc\nu/kT)$, then $T = 1{,}074°$ K. This deviation seems well outside both the experimental errors and the uncertainty in measurement of the oven temperature,[1] 805 ± 100° K., and the vibrational frequency,[4] 715 ± 15 cm^{-1}. Corrections for the background from scattered molecules and losses from the beam and ion currents do not discriminate between the vibrational states. Saturation of the r.f. resonances could cause errors which differ with v, and care was taken to avoid this.[5] Although the calculation of N_v was based upon the assumption that the molecules leaving the oven are harmonic oscillators in thermal equilibrium with respect to vibration, there would have to be a very large deviation to cause a discrepancy comparable with the ratio found in equation (2). Translational equilibration is of minor importance, since this enters only in computing the small radiative correction.

The strong increase of I_v/N_v with v therefore will be interpreted to mean that the detecting reaction depends upon the vibrational state of the incident lithium chloride molecules. Equation (2) is then the relative rate constant for the over-all reaction (1) as a function of the initial vibrational state.

Several kinds of evidence[6] indicate that reaction (1) occurs by dissociation of the alkali halide molecule into atoms, followed by ionization of the alkali atom. The dependence of the net reaction on the vibrational state of the incident molecules is then to be sought in the probability of capture by the surface, the residence period on the surface, and the dissociation process.

Molecular properties such as the dipole moment and polarizability which evidently are responsibile for the attraction to the surface vary slowly with vibrational state. It is therefore unlikely that the capture probability could change significantly with vibrational state.

If almost every molecule striking the surface were to stick until it dissociated, any dependence of the reaction on initial state would be ineffective. Thus, in order for I_v/N_v to depend upon v, it is necessary that a significant fraction of the molecules evaporate from the wire before they dissociate.

Molecules incident in excited vibrational states have a kinetic advantage because the average time required to attain the dissociation energy decreases with increasing initial vibrational energy. A smaller fraction is therefore lost by evaporation before dissociation, so that I_v/N_v is expected to increase with v. Thus the evaporation rate effectively defines a time scale with which to measure the progress of the energy-transfer processes leading to dissociation.

Model for the Reaction.—We now adopt a simple phenomenological model for the reaction process to illustrate the above considerations and to obtain a quantitative comparison with equation (2). The basic assumption is that the internal motions of adsorbed molecules are but slightly affected by interaction with the surface. The wire is regarded as an energy bath and the transfer of energy treated as in radiation theory and gas kinetics. For a weak interaction model it is readily shown that the most important energy-transfer processes involve unit changes of the vibrational quantum number and that the evaporation rate constant is sensibly independent of vibrational state.

Figure 2 is a schematic diagram of the model. The molecular-beam apparatus sends molecules in one of the four states $v_{\text{initial}} = i = 0, 1, 2,$ or 3 onto the wire

at a steady rate. The adsorbed molecules are distributed over vibrational states by energy exchange with the wire. Molecules in states of sufficient excitation energy dissociate and then ionize, giving a steady ion current. (For order-of-magnitude comparison, the dissociation energy of a free molecule is 41,160 cm^{-1}, while $\nu = 715\ cm^{-1}$ and the wire at 1,400° K. has a mean energy $kT = 970\ cm^{-1}$.)

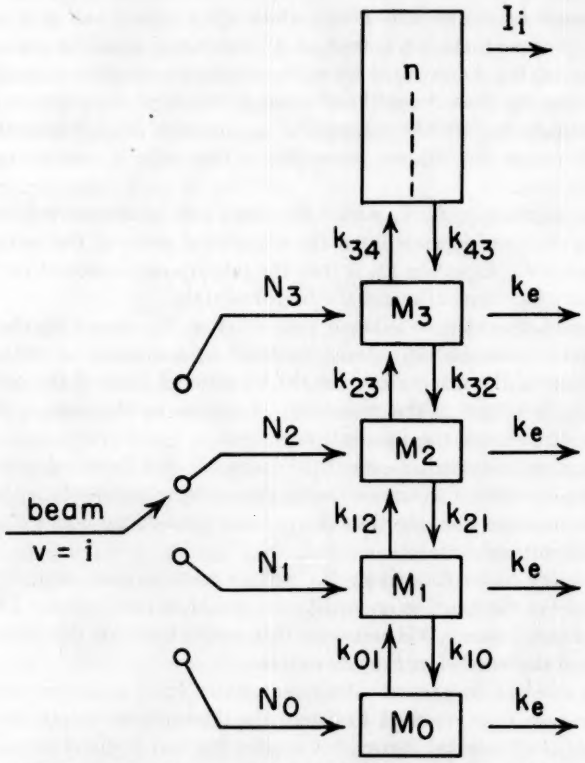


FIG. 2.—Schematic representation of the model for the reaction.

Detailed balancing of the competing processes defines the steady-state distribution of populations, $M_v(i)$. The rate of supply to the initial state is $k_s N_i$, where k_s depends upon the apparatus but not on i, since the probability of capture by the surface does not depend upon vibrational state. The rate of transitions from state v to $v + 1$ is $k_{v,\,v+1}M_v$, and $k_{v+1,\,v}M_{v+1}$ is the rate of the inverse process. The rate of evaporation is $k_e M_v$. The steady-state condition for state v is then

$$\frac{dM_v}{dt} = 0 = k_s N_i \delta_{vi} + k_{v+1,\,v}M_{v+1} + k_{v-1,v}M_{v-1} - (k_e + k_{v,\,v+1} + k_{v,\,v-1})M_v, \tag{3}$$

where $\delta_{vi} = 1$ if v is the initial state i, the state being supplied by the beam, and zero otherwise.

The populations $M_v(i)$ are obtained as solutions of the array of coupled linear equations

$$\begin{array}{cccccccc} v'v & 0 & 1 & 2 & 3 & 4 \ldots & & \\ 0 & A_0 & k_{10} & 0 & 0 & 0 & = C_0 & \\ 1 & k_{01} & A_1 & k_{21} & 0 & 0 & = C_1 & \\ 2 & 0 & k_{12} & A_2 & k_{32} & 0 & = C_2 & \\ 3 & 0 & 0 & k_{23} & A_3 & k_{43} & = C_3 & (4) \\ 4 & 0 & 0 & 0 & k_{34} & A_4 & = 0 & \\ \cdot & & & & & \cdot & \cdot & \\ \cdot & & & & & \cdot & \cdot & \\ \cdot & & & & & \cdot & \cdot & \end{array}$$

where $-A_v = k_e + k_{v,\,v+1} + k_{v,\,v-1}$ and $C_v = -k_v N_i \delta_{vi}$; C_4 and all higher C_v are zero in the present experiment.

The populations of all states having $v > i$ are proportional to $M_i(i)$, but the population ratios are independent of i. As in Figure 2, the states above the highest state studied experimentally, $v = 3$, are considered collectively and designated "states n." Their populations are proportional to $M_3(i)$. The states which directly dissociate are among "states n," and therefore the ion current may be expressed as

$$I_i = K_n M_3(i), \tag{5}$$

where K_n is independent of i. The comparison with experiment is then obtained from

$$(I_i/N_i)_{\text{calculated}} = \frac{M_3(i)/N_i}{M_3(0)N_0}. \tag{6}$$

There are several relations among the rate constants which permit the evaluation of equation (6) in terms of one parameter. For excitation of molecular vibrations induced by the lattice vibrations of the surface,

$$k_{v,\,v+1} = (v + 1)k_{01} = (v + 1)k, \tag{7}$$

in the harmonic oscillator approximation; k is determined by properties of the molecule as well as by properties of the surface. The rate constant for the inverse process $k_{v+1,\,v}$ refers to both induced and spontaneous transfer of energy from the molecule to the surface and is given, as in radiation theory, by

$$\frac{k_{v,\,v+1}}{k_{v+1,\,v}} = f = \exp\left(-\frac{hc\nu}{kT}\right) \tag{8}$$

For $\nu = 715\ \text{cm}^{-1}$ and $T = 1{,}400 \pm 200°$ K. the Boltzmann factor $f = 0.48 \pm 0.05$. For the state $i = 1$, equation (6) is simply $1 + k_e/k$. We set $k_e/k = 0.4$ to fit experiment. The predictions for the two remaining states are given in Table 1. Since these are directly dependent upon the harmonic oscillator model used and also upon the value of f, the agreement with experiment is considered quite satisfactory.

In order to obtain the populations of states below $v = 3$, it is only necessary that the harmonic oscillator approximation and its consequent one quantum selection

rule extend to a few higher states. The uncoupling of equations (4) is effected by using the rapidly converging continued fraction

$$\frac{M_v}{M_{v-1}} = \cfrac{-k_{v-1,\,v}}{A_v - \cfrac{k_{v,\,v+1}k_{v+1,\,v}}{A_{v+1} - \cfrac{k_{v+1,\,v+2}k_{v+2,\,v+1}}{A_{v+2} - \ldots}}}, \quad \text{for } v > i. \tag{9}$$

Since $k_{v,\,v+1}$ increases with v, evaporation becomes less important at high v; it may be shown from equation (9) that the populations approach the equilibrium thermal distribution, $M_v/M_{v-1} \rightarrow f$, either as v increases or as $k_e \rightarrow 0$. The steady-

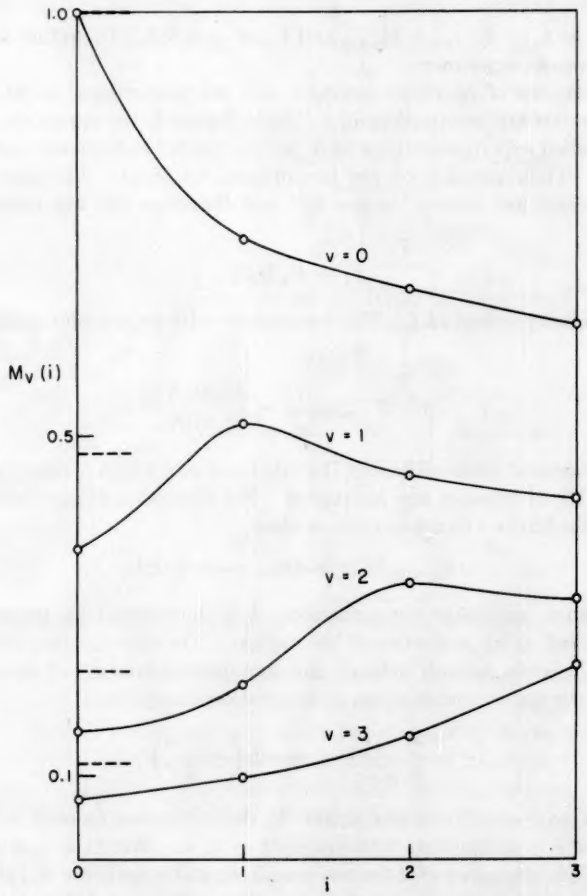


FIG. 3.—Approximate distribution of steady-state vibrational populations, $M_v(i)$, for different choices of the incident state. For $k_e/k = 0.4$, $f = 0.48$ (1,400° K.), and normalized to $N_i = 1$, $M_0(0) = 1$. The dotted lines mark the equilibrium values if there is no evaporation and no incident molecules.

state distributions as functions of the state of the incident molecules are shown in Figure 3.

We emphasize that the idealizations of the model may be relaxed as v increases without affecting the calculations made for the lowest few vibrational levels. All states above $v = 3$ have been considered collectively in the calculation of the ion currents, to point out that the present experiment gives no information about processes involving these higher states and that its analysis therefore does not require detailed consideration of these states.

We have enjoyed discussions with Professor J. W. Trischka and Professor N. F. Ramsey and wish to thank them. We are especially grateful to Professor G. B. Kistiakowsky for the considerable help and encouragement he has given us.

* Charles A. Coffin Fellow, 1956–1957.

[1] D. T. F. Marple and J. W. Trischka, *Phys. Rev.*, **103**, 597, 1956.
[2] J. W. Trischka, private communication.
[3] J. A. Becker and R. G. Brandes, *J. Chem. Phys.*, **23**, 1323, 1955.
[4] W. Klemperer and S. A. Rice, *J. Chem. Phys.*, March, **26**, 618, 1957.
[5] R. G. Luce and J. W. Trischka, *J. Chem. Phys.*, **21**, 105, 1953.
[6] S. Datz and E. H. Taylor, *J. Chem. Phys.*, **25**, 395, 1956, and earlier references cited therein.

INFLUENCE OF NOTOCHORD ON DIFFERENTIATION OF STRIATED MUSCLE IN AMBYSTOMA

BY WILLIAM B. MUCHMORE

DEPARTMENT OF BIOLOGY, UNIVERSITY OF ROCHESTER, ROCHESTER, NEW YORK

Communicated by J. Holtfreter, February 25, 1957

Much has been said in recent years regarding the influence of the notochord upon the differentiation of various other tissues in early embryos of amphibians. Following Yamada,[1–3] other authors[4–7] have adopted the view that the developing notochord is in some way responsible for the establishment and maintenance of a dorsoventral gradient of "morphogenetic potential" within the embryo. This gradient is expressed in the mesodermal mantle of the trunk as the dorsal-to-ventral sequence of differentiation—myotome, pronephros, lateral plate, and blood cells. It is generally implied that the gradient results from the diffusion out from the notochord of some factor which has been termed "chorda-factor."

If some factor from the notochord is indeed necessary for the direction of differentiation of the trunk mesoderm, including the striated muscle, then, in general, the following results would be expected: (*a*) when the notochord is removed from early stages, muscle differentiation should be markedly inhibited; (*b*) when notochord is explanted with presumptive muscle tissue (viz., somite) muscle differentiation should tend to be normal; and (*c*) when notochord is explanted together with presumptive pronephros, induction of muscle differentiation might be expected.

It has already been shown that result *a* is not realized, for when notochord is removed from early neurulae of *Ambystoma*, differentiation of the muscle masses is little affected.[8–11] The notochord is obviously not necessary for muscle differentiation *in situ*.

In regard to the second and third points (*b* and *c*), it is true that explantation of somite tissue or lateral mesoderm of early neurulae together with presumptive notochord has usually resulted in the production of large amounts of muscle tissue[1, 11, 12] However, it has also been demonstrated that presumptive somite explanted into ectodermal envelopes can self-differentiate into muscle in the complete absence of notochord if the mass of the explant is large enough (Muchmore[13]). Furthermore, some doubt as to the origin of the muscle tissue in the combination explants has been aroused by the demonstration that presumptive notochord explanted alone can form much muscle tissue (Muchmore[11]). At the same time it was found that the muscle-forming potentiality of the notochord seemed to be localized in the lateral edges; that is, the medial third of the presumptive notochord formed only notochord, while the lateral thirds yielded both notochord and muscle. These results created the need to repeat the explantation experiments using only the medial portion of the notochord.

Early neurulae (Harrison's stage 13) of *Ambystoma maculatum* were used in all the experiments reported here. The anterior four-fifths of the presumptive notochord was excised, together with the overlying neural plate. Then the lateral thirds of this long strip were cut off and discarded, and the neural material was separated from the medial third of the presumptive notochord. Great care was taken to insure a clean isolation of this notochordal strip. Presumptive myotome (somite) and presumptive pronephros were isolated from the areas of the neurula indicated in an earlier work.[11] Ventral, mesoderm-free ectoderm from the same embryos was used to envelop the explanted tissues. The following combinations of primordia were made: (1) notochord alone; (2) notochord plus somite; and (3) notochord plus pronephros.

Histological examination of the explants yielded the data summarized in Table 1. In every case the notochord was a well-differentiated cylinder or mass, and it was generally of large size.

TABLE 1

DIFFERENTIATIONS WITHIN THE EXPLANTS

Series	Total No.	Notochord	Muscle	Nephric Tubules	Blood Cells	Mesenchyme	Neural Tissue	Undiff. Cells
1. Notochord alone	56	56	36	0	0	47	37	6
Per cent		*100*	*64*	*0*	*0*	*84*	*66*	*11*
2. Somite plus notochord	53	53	45	50	0	53	17	53
Per cent		*100*	*85*	*94*	*0*	*100*	*32*	*100*
3. Pronephros plus notochord	51	51	19	37	4	50	25	48
Per cent		*100*	*37*	*75*	*8*	*98*	*49*	*94*

In a great number of cases, the presumptive notochord, explanted alone (series 1), gave rise to striated muscle cells, usually small in amount. Differentiation of muscle in these explants may have been due to the larger amount of medial notochord employed (as compared with the previous explants of Muchmore[11]) or to a greater potentiality for muscle differentiation in the posterior part of the presumptive notochord. Whatever the origin of these muscle cells, it might be expected that a similar proportion of the muscle found in the other experimental series has developed from the same source. If so, any muscle produced in excess of this amount may be considered to have been induced by the notochord.

The neural structures encountered are of varied shapes and sizes, ranging from small, solid masses to large, irregular, vesicular structures. Most, however, tended to be small in size. The neural tissue has probably originated from the anterior edge of the ventral ectoderm, which in the neurula lies very close to the forebrain and nasal fields.

In series 2 (somite plus notochord) muscle differentiation was somewhat more frequent than in the first series, and nephric tubules occurred in a large number of cases. Together with the fact that presumptive somite, when explanted alone, gives rise to muscle and pronephros in very few cases (cf. Muchmore[11]), this indicates that notochord has had some effect on the differentiation of these other tissues. This effect is, however, not nearly so great as might have been expected on the basis of previous work which has suggested that the notochord is strongly inductive. Furthermore, in the third series the presence of well-differentiated notochord has not resulted in any increase in the differentiation of muscle; indeed, a decrease seems evident. Although the presumptive pronephros, explanted alone, would have produced little or no differentiated pronephric tubules, the tubule production in this third series of explants is also below that which might be expected in the presence of a powerful "chorda-factor," operating to raise the general "morphogenetic potential."

The high frequency of muscle in the second series is perhaps less significant than appears from Table 1. In each explant containing muscle cells, the relative amount of muscle as compared to other mesodermal elements (nephric tubules, blood cells, undifferentiated cells) was estimated as trace (±), up to 25 per cent (+), 25–50 per cent (++), 50–75 per cent (+++), and 75–100 per cent (++++). Because of the jumbled relations of the tissues in explants, an exact measure of the quantity of any one tissue is impossible, but the estimates were made with great care and in the same manner throughout.

From Table 2 it can be seen that the distribution of quantity of muscle is similar in all three series; that is, whenever muscle did turn up, it occurred in relatively small amounts and in nearly the same proportions in all series (the values in the

TABLE 2

QUANTITATIVE OCCURRENCE OF MUSCLE TISSUE IN THE EXPLANTS

SERIES	NO. WITH MUSCLE	QUANTITY OF MUSCLE				
		±	+	++	+++	++++
1. Notochord alone	36	2	31	3	0	0
2. Somite plus notochord	45	4	36	5	0	0
3. Pronephros plus notochord	19	5	12	2	0	0

three series are statistically homogeneous at the 20 per cent level). Addition of notochord to presumptive somite and pronephros has not resulted in the formation of larger amounts of muscle tissue than was formed by presumptive notochord alone.

These results seem to indicate that the medial portion of the presumptive notochord from *Ambystoma neurulae*, while able to differentiate into notochord, is not a potent inductor of muscle differentiation in somite or lateral mesoderm. In other words, *notochord in itself* is not effective in bringing about muscle differentiation. This lends further support to the idea, expressed previously,[13] that presumptive

somite of the neurula can differentiate into muscle and pronephros under relatively nonspecific, but favorable, conditions. Then such "excess" differentiations as are seen in series 2 are considered to be due to the simple physical presence of the expanding notochord, preventing dispersal of the somite cells, or to the provision by the notochord of some other very general requirement(s) for the development of these tissues.

Statistical analysis of the preceding experiments revealed rather unexpectedly that muscle, pronephric tubules, and nervous tissue seem to differentiate independently of one another. For example, in the first series (notochord explanted alone) muscle occurred in 36 and neural tissue in 37 of the 56 cases, that is, in 64.3 and 66.1 per cent of the total, respectively. These values are used to set up a 2×2 contingency table based on the assumption that muscle and neural tissue are independent (Table 3). The chi square, calculated from the deviations of the observed from the expected values, is 2.558, with p slightly greater than 0.1, indicating agreement with the hypothesis of independence.

TABLE 3

2×2 CONTINGENCY TABLE FOR THE INDEPENDENT OCCURRENCE OF MUSCLE (M) AND NEURAL TISSUE (N) IN EXPLANTS OF PRESUMPTIVE NOTOCHORD ALONE

(Figures in Parentheses Are Expected Values Calculated from Border Totals)

	M Present	M Absent	Total
N present	27 (23.80)	10 (13.22)	37
N absent	9 (12.21)	10 (6.78)	19
Total	36	20	56

Using Yates's adjustment, $\chi^2 = 2.558$
d.f. $= 1$; $0.2 > p > 0.1$

In a similar fashion, the data from the second and third series can be used to set up $2 \times 2 \times 2$ contingency tables for the independent occurrence of muscle, pronephric tubules, and neural tissue. In the second series (Table 4*a*) the p of 0.7 demonstrates agreement with the hypothesis of independence. But in the third series (Table 4*b*) it would appear that there is some association among the tissues, particularly between muscle and neural tissue. It is unlikely, however, that the probabilities given in the published tables of chi square are applicable to the present situation, where several of the expected values are smaller than 5. In cases with small expected values, the 5 per cent level of significance would refer to larger values of chi square than are shown in the tables; thus the large chi square in this instance may not be significant. As a matter of fact, when chi square is calculated for M and N alone, ignoring P, no significant deviation from the expected values is found, chi square being 1.610, with $p = 0.2$. Thus, under the conditions existing in these explants, muscle, pronephric tubules, and neural tissue may be considered to develop independently.

The results presented above lend further support to the idea that no potent inductor of mesodermal structures is operating here, for if a "chorda-factor" were actually influencing the differentiation of both muscle and nephric tubules, then these tissues should be expected to occur together much more frequently than

TABLE 4

2 × 2 × 2 CONTINGENCY TABLES FOR THE INDEPENDENT OCCURRENCE OF MUSCLE (M), PRONEPHRIC TUBULES (P), AND NEURAL TISSUE (N)
(Figures in Parentheses Are Expected Values Calculated from Border Totals)

(*a*) IN EXPLANTS OF PRESUMPTIVE SOMITE AND NOTOCHORD

	M PRESENT		M ABSENT		TOTAL
	N Present	N Absent	N Present	N Absent	
P present	14 (13.62)	29 (28.81)	2 (2.42)	5 (5.13)	50
P absent	1 (0.82)	1 (1.74)	0 (0.15)	1 (0.31)	3
Total	15	30	2	6	53

$\chi^2 = 2.128$
d.f. = 4; $0.75 > p > 0.70$

(*b*) IN EXPLANTS OF PRESUMPTIVE PRONEPHROS AND NOTOCHORD

	M PRESENT		M ABSENT		TOTAL
	N Present	N Absent	N Present	N Absent	
P present	5 (6.94)	6 (7.23)	11 (11.67)	16 (12.15)	38
P absent	7 (2.38)	1 (2.47)	2 (4.00)	3 (4.16)	13
Total	12	7	13	19	51

$\chi^2 = 13.133$
d.f. = 4; $0.02 > p > 0.01$

they would were their combination due to chance alone. The data support the latter view and suggest that in the explanted presumptive somitic material there are separate "centers" for the differentiation of muscle and of pronephros which come into operation independently of one another. This interpretation conforms to the idea of separate but overlapping morphogenetic fields for the various mesodermal anlagen as outlined previously (Muchmore[11]).

Summary.—Presumptive notochord, somite, and pronephros, in several combinations, were explanted from early neurulae of the salamander *A. maculatum*. The results support the idea that the notochord is not an efficient inductor of muscle and nephric tubules. Statistical analysis of the data suggests that muscle, nephric tubules, and neural tissue differentiate independently of one another and that they are not mutually dependent upon an influence by the notochord. The experiments are interpreted as indicating the presence, in the neurula, of independent morphogenetic fields.

[1] T. Yamada, *Folia Anat. Japon.*, **19**, 131, 1940.
[2] T. Yamada, *Embryologia*, **1**, 1, 1950.
[3] T. Yamada, *Biol. Bull.*, **98**, 98, 1950.
[4] S. M. Rose, *Am. Naturalist*, **86**, 337, 1952.
[5] Y. Hayashi, *Embryologia*, **2**, 145, 1955.
[6] A. Kühn, *Vorlesungen über Entwicklungsphysiologie* (Berlin, 1955).
[7] C. H. Waddington, *Principles of Embryology* (London, 1956).
[8] S. Hörstadius, *Acta zool.*, **25**, 75, 1944.
[9] P. D. Nieuwkoop, *Arch. néer. zool.*, **8**, 1, 1946.
[10] I. C. Kitchin, *J. Exptl. Zool.*, **112**, 393, 1949.
[11] W. B. Muchmore, *J. Exptl. Zool.*, **118**, 137, 1951.
[12] T. Yamada, *Folia Anat. Japon.*, **18**, 565, 1939.
[13] W. B. Muchmore, *J. Exptl. Zool.*, Vol. **134**, 1957 (in press).

*A STUDY OF ASYMMETRY IN THE DEVELOPING GUT**

By Sally Wilens

OSBORN ZOOLOGICAL LABORATORY, YALE UNIVERSITY

Communicated by J. S. Nicholas, March 15, 1957

While movements of the surface materials of the amphibian egg, prior to gastrulation, have been mapped with the use of vital staining technique,[1, 2] the migrations of the inner-layer components of later stages, related to organogenesis, have been charted less extensively. To study the movements of the endoderm, Nile Blue Sulphate was applied to various ventral levels of young embryos of *Amblystoma punctatum* (Harrison's stages 14–15, wide-open medullary plate). The following regions were stained (Fig. 1): (1) endoderm in the heart level—a short distance from the forebrain region, no mesoderm present at time of staining; (2) midventral region—no mesoderm present at time of staining; (3) ventrolateral regions—in the same level as region 2; (4) posterior regions—anterior and posterior trunk.

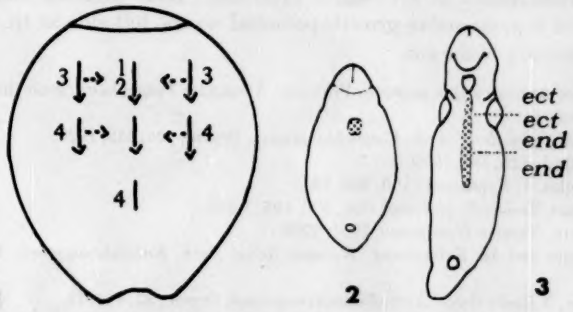
Method.—A fine incision was made in the ventral body wall of the living embryo, and a small piece of stained cellophane was inserted for 2 or 3 minutes. After removal of the cellophane, healing was rapid. The incisions were transverse or longitudinal, depending on the location of stain desired in a given region. In dilute Holtfreter's solution there was no loss of material, and staining was effective.

Advantage was taken of three characteristics of vitally stained endoderm: first, its propensity for taking up the stain, described by Nicholas[3] in the study of the blastula; second, the persistence of the stain; and, third, its visibility in the ventral wall of the live embryo. The present technique makes it possible to study the layers that are in contact (ectoderm and endoderm) at the time of staining, thus permitting observation of their relationship during progressive development. A series of camera lucida drawings was made of each living embryo to record the position of the stain at various stages.

Results.—As the embryo develops, the stained patches stretch posteriorly in a thin strip. The initial placement of the stains and the direction of movement are indicated in Figure 1. Stain taken up by the overlying ectoderm extends a short distance; the stained endoderm can be seen to continue posteriorly beyond the stained ectoderm (Figs. 2 and 3). The extension of the stained endoderm was confirmed in section. The ventrolateral stains (left and right sides) at first stretch posteriorly, then converge and move toward the midventral region, where fusion of the two stains occurs in the ventral wall of the gut. When the ventral endodermal substrate of the prospective heart region is stained at stage 14, prior to the invasion of the lateral mesoderm, the blue stain is found in the ventral wall of the pharynx in contact with unstained heart tissue. Stain applied to the posterior part of the trunk shows very little posterior stretch and remains in the position where originally placed.

The persistence of the stain in later stages makes it visible in the ventral wall of the developing gut in the living embryo. Stain applied to the midline (ten cases) and the right ventrolateral side (three cases), a short distance posterior to the forebrain region, was found in the right wall of the duodenum, the region of first curvature. The asymmetry of the gut was normal in all these embryos. When

ventrolateral stains were applied on the right and left sides, inverse symmetry, i.e., situs inversus viscerum, was found in two cases. In one case, stained on the left side, there was absence of curvature in the duodenal region, and the pancreas was displaced toward the right side posterior to two large, nonconnecting gall bladders; another case stained similarly showed situs inversus viscerum. Two embryos stained in the midventral region showed reversal of asymmetry. In these six cases the blue stain was found in the left wall of the duodenum in section.



FIGS. 1–3.—Fig. 1, diagram of ventral view of an embryo at stage 15. Stain was applied to regions numbered *1–4;* arrows indicate direction of movement of stained endoderm. Fig. 2, camera lucida drawing of ventral view of embryo, stage 21 (STEND 100, stained at stage 14). Patch of ectoderm and underlying endoderm stained with Nile Blue Sulphate (*stippled area*). Fig. 3, camera lucida drawing, ventral view of the same embryo, stage 33. Thin strip of stained endoderm has stretched posteriorly beyond the ectoderm (*stippled area*); *ect.* = ectoderm; *end.* = endoderm.

Discussion.—In the cases in which stain was applied to the left side in the ventral wall of the embryo and later localized in the duodenum, inverse symmetry of the duodenum with irregularity in the position of the gall bladder and pancreas resulted. This method of obtaining reversal of symmetry contrasts very sharply with the methods used by many investigators which involved interference with development on the dorsal side of the embryo. Spemann[4, 5] and Pressler[7] rotated a section of the neural plate and archenteric roof of the anuran embryo, young neurula stage, and obtained inverse symmetry. Spemann and Falkenberg[6] after mechanical constriction in the blastula stage, found situs inversus to occur in the right-hand member of the twins and double monsters of the newt. Wilhelmi[8] removed pieces of the neural plate and archenteric roof on the left side at various levels and observed situs inversus in a few cases, but failed to test the right side of the embryo. Dalcq[9] interchanged regions of the dorsal side of the anuran embryo at various stages and obtained situs inversus in some of his experiments. Experiments performed on the ventral side of the embryo permit direct interference with the process of embryonic gut development. The complicating effects of rotating the nervous system with the archenteric roof, as well as those of transplantation and defect experiments, are thus eliminated. The vital stain serves to label the region affected by the operation.

Interference in the left ventral side of the embryo in the level of the duodenum produces situs inversus viscerum. This is the region of first curvature of the

gut, resulting from the shift to the right side, of tissue derived from the left side of the embryo. Rightness and leftness of derivation are maintained and can be observed in later development when these regions have been stained. When stain was localized in the right wall of the duodenum after application to the right or midventral region in the level of the prospective duodenum, or posterior to this level, the asymmetry of the gut and associated structures was perfectly normal.

Interference with normal development on the left side in the region of the prospective duodenum results in reversal of symmetry (situs inversus viscerum). The dominance of a more active growth potential on the left side at this level insures normal asymmetry of the gut.

* The author dedicates this paper to Professor Alexander Petrunkevitch on the occasion of his eightieth birthday.

[1] W. Vogt, *Wilhelm Roux' Arch. Entwicklungsmech. Organ.*, **106**, 542, 1925.
[2] W. Vogt, *ibid.*, **120**, 384, 1929.
[3] J. S. Nicholas, *J. Exptl. zool.*, **100**, 265, 1945.
[4] H. Spemann, *Verhandl. deut. zool. Ges.*, Vol. **195**, 1906.
[5] H. Spemann, *Naturw. Rundschau*, **21**, 1, 1906.
[6] H. Spemann and H. Falkenberg, *Wilhelm Roux' Arch. Entwicklungsmech. Organ.*, **45**, 371, 1919.
[7] K. Pressler, *Wilhelm Roux' Arch. Entwicklungemech. Organ.*, **32**, 1, 1911.
[8] H. Wilhelmi, *Wilhelm Roux' Arch. Entwicklungsmech. Organ.*, **48**, 517, 1921.
[9] A. Dalcq, *Acta Anat.*, **4**, 100, 1947.

ERRATA: PARABOLIC SYSTEMS OF DIFFERENTIAL EQUATIONS WITH TIME DEPENDENT COEFFICIENTS

In the article of the above title appearing in these PROCEEDINGS, **42**, 914–917, 1956, the following correction should be made:

C as defined in the paragraph following equation (1.6) is not compact. The proof of Theorem 1 is still valid, however, if one modifies the definition of the inner product $[\ ,\ ]$, setting

$$[\phi, \psi] = (-1)^m[\phi, \psi]_{A_1} + \lambda(B\phi, \psi) + {}^1/_2[\phi, \psi]_{B_1}.$$

Then, after the substitution $u = ve^{-\lambda t}$ with λ large and positive, S defined as before, $C = 0$, the result of Lemma 1 is applicable.

FELIX E. BROWDER

INFORMATION TO CONTRIBUTORS

THE PROCEEDINGS is the official organ of the NATIONAL ACADEMY OF SCIENCES and of the NATIONAL RESEARCH COUNCIL for the publication of brief accounts of important current researches of members of the ACADEMY and of the COUNCIL and of other American investigators. The PROCEEDINGS will aim especially to secure prompt publication of original announcements of discoveries and wide circulation of the results of American research among investigators in other countries and in all branches of science.

ARTICLES should be brief. The viewpoint should be comprehensive in giving the relation of the paper to previous publications of the author or of others and in exhibiting, where practicable, the significance of the work for other branches of science. Elaborate technical details of the work and long tables of data should be avoided, but authors should be precise in making clear the new results and should give some record of the methods and data upon which they are based.

MANUSCRIPTS should be prepared with a current number of the PROCEEDINGS as a model in matters of form, and should be typewritten in duplicate with double spacing, the author retaining one copy. Illustrations should be confined to text figures of simple character, though more elaborate illustration may be allowed in special instances to authors willing to pay for their preparation and insertion. Particular attention should be given to arranging tabular matter in a simple and concise manner.

MANUSCRIPTS will be accepted only from members of the Academy who will assume responsibility for the propriety and scientific standards of the paper and for any printing costs in excess of those allowed them by the Academy. Manuscripts should be addressed to *Proceedings of the* NATIONAL ACADEMY OF SCIENCES, Attention Miss Mary D. Alexander, University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois.

GALLEY PROOF will be sent, with the understanding that charges for author's corrections shall be billed to him. Author should therefore make final revisions on the typewritten manuscripts. Page proofs will not be sent.

REPRINTS should be ordered when proof is returned, on the reprint order form which will be inclosed with the proof. The order blank gives a tabular estimation of the cost of reprints; a copy will be supplied at any time upon request.

CONTENTS

Page

BIOCHEMISTRY.—THE OXIDATION OF REDUCED TRIPHOSPHOPYRIDINE NUCLEOTIDE AS MEDIATED BY THE TRANSHYDROGENASE REACTION AND ITS INHIBITION BY THYROXINE. . . . . *By Eric G. Ball and Octavia Cooper* 357

BIOCHEMISTRY.—INTERRELATIONSHIP BETWEEN VITAMIN E AND LIPIDE COFACTOR IN THE CYTOCHROME C REDUCTASE SYSTEM . . . *By Kenneth O. Donaldson and Alvin Nason* 364

BIOCHEMISTRY.—MEIOTROPHIC MUTANTS OF *Pasteurella Pestis* AND THEIR USE IN THE ELUCIDATION OF NUTRITIONAL REQUIREMENTS . . . . . *By Ellis Englesberg and Laura Ingraham* 369

BIOCHEMISTRY.—COMPETITIVE BINDING OF ATP AND ACRIDINE ORANGE BY MUSCLE . . . . . *By George Karreman, Helmut Mueller, and Albert Szent-Györgyi* 373

BIOCHEMISTRY.—THE BEHAVIOR OF TWO FOREIGN PROTEIN ANTIGENS IN MICE DURING INHIBITION OF ANTIBODY FORMATION BY CORTISONE . . . . . *By Philip D. McMaster and Joshua L. Edwards* 380

BIOCHEMISTRY.—PHOTO SPIN RESONANCE IN CHLOROPHYLL-CONTAINING PLANT MATERIAL . . . . . *By Power B. Sogo, Ning G. Pon, and Melvin Calvin* 387

BIOCHEMISTRY.—BIOCHEMICAL BASIS FOR THE TOXICITY OF PENTACHLOROPHENOL. . . . . *By Eugene C. Weinbach* 393

BOTANY.—EVIDENCE FOR "GIBBERELLIN-LIKE" SUBSTANCES FROM FLOWERING PLANTS. . . . . *By Bernard O. Phinney, Charles A. West, Mary Ritzel, and Peter M. Neeley* 398

GENETICS.—THE EFFECT OF HETEROZYGOSITY FOR NEW MUTATIONS ON VIABILITY IN *Drosophila:* A PRELIMINARY REPORT . . . . . *By Bruce Wallace* 404

MATHEMATICS.—SPHERICAL FUNCTIONS ON A SEMISIMPLE LIE GROUP *By Harish-Chandra* 408

MATHEMATICS.—ON THE HOMOLOGY OF $K(\pi, n)$ . . . . . *By John C. Moore* 409

MATHEMATICS.—THE DIVERGENCE THEOREM WITHOUT DIFFERENTIABILITY CONDITIONS. . . . . *By Victor L. Shapiro* 411

PHYSICS.—DIFFUSION OF PARTICLES WITH MEMORY . . . . . *By Peter L. Bender* 412

PHYSICS.—CODES WITHOUT COMMAS . . *By F. H. C. Crick, J. S. Griffith, and L. E. Orgel* 416

PHYSICS.—STRAIN ELECTROMETRY AND CORROSION I. GENERAL CONSIDERATIONS ON INTERFACIAL TRANSIENTS . . . . . *By Albert G. Funk, J. Calvin Giddings, Carl J. Christensen, and Henry Eyring* 421

PHYSICS.—VARIATION OF REACTION RATE WITH VIBRATIONAL STATE . . . . . *By William Klemperer and Dudley Herschbach* 429

ZOÖLOGY.—INFLUENCE OF NOTOCHORD ON DIFFERENTIATION OF STRIATED MUSCLE IN *Ambystoma* . . . . . *By William B. Muchmore* 435

ZOÖLOGY.—A STUDY OF ASYMMETRY IN THE DEVELOPING GUT . . . . *By Sally Wilens* 440

ERRATA.—PARABOLIC SYSTEMS OF DIFFERENTIAL EQUATIONS WITH TIME DEPENDENT COEFFICIENTS . . . . . *By F. E. Browder* 442